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CHANGES IN THE ESTERASE AND FAT CONTENT OF THE SERUM INDUCED BY CANCER AND CANCER-PRODUCING AGENTS.

HARRY NORMAN GREEN.

From the Department of Pharmacology, University of Sheffield.

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IN the course of an investigation on the serum esterases in vitamin A deficiency (Green, 1934), a rat bearing a large Jensen sarcoma, but active and otherwise apparently healthy, was used as a control. The blood-serum was noted to be very milky in appearance and its content of esterase was almost negligible. This observation led to a more detailed study of the serum esterase and fat in the cancerous animal, particularly in the serum of the rat inoculated with the Jensen sarcoma.

Previous work on lipolytic enzymes in cancer appears to have originated in the observations of Shaw-Mackenzie (1915), who found that the power of normal serum to accelerate the hydrolysis of olive oil by a glycerol extract of pancreas was definitely diminished in cases of human carcinoma and in certain other pathological conditions. He had previously shown (1911) that the power of the serum to activate lipase was increased in mice which had recovered spontaneously from large growths, and in mice resistant to implanted grafts or rendered immune by injection of normal tissues. The observations in human cancer were confirmed by Corran and Lewis (1928), using a more refined technique, but Woodhouse (1933), who prepared an entirely inactive specimen of pancreatic prolipase, was unable to find any difference between normal and cancerous sera in their activating effect on prolipase. The significance of the earlier work is therefore somewhat obscure at present.

Little attention has been paid to possible alterations in the power of the serum itself to hydrolyse the lower fatty esters as distinct from its property of activating pancreatic prolipase. Falk and co-workers (1924) carried out extensive observations on the esterase content of tumour tissue (Flexner-Jobling rat carcinoma), and ascribed to it a curve for its relative action on a series of lower fatty acid esters differing from the characteristic curves found

for various normal tissues. They also found (1925) that the curve obtained from tissues of embryos and very young rats approached nearest in form to that of the carcinoma tissue. Work more closely related to that of the present investigation was reported by Begg and Aitken (1932), who found that the capacity of the serum to hydrolyse tributyrin was greater than normal in fowls cured of a Rous sarcoma by bleeding, in fowls immune to cell inoculations of Rous sarcoma, and in various animals after injection with surviving foreign tumour tissue. Only a few results are reported, and in such (though no comment is made by the authors) there is no indication of a decided change in the serum esterase content due to the presence of a growing sarcoma.

Investigations on the blood fats and lipoids in cancer, though numerous, have been mainly inconclusive. Rémond and co-workers (1925) noted an increase in the fatty acids, cholesterol and phospholipines of the blood in the precancerous stage of the tar-painted rabbit, followed by an abrupt fall at the onset of malignancy and a gradual rise to normal. Mattick and Buckwald (1929) observed a tendency to hypercholesteræmia in the plasma, and an increase in the total fatty acids of the plasma in human cancer. Irregular changes in the cholesterol content of the blood in experimental and human cancer have been noted by many workers, but no definite conclusions have been reached.

EXPERIMENTAL METHODS.

Young, or fairly young white or piebald rats were inoculated with a strain of Jensen sarcoma. At various stages of tumour growth blood was obtained by severing the neck vessels after previous stunning with a head blow. The blood was immediately centrifuged and the serum used within a maximum period of 6 hours.

All the rats received a stock diet, which was unaltered in any of the experiments.

The serum esterase activity was estimated by the following method : To 20 c.c. of M/80 phosphate buffer of pH 8·0 measured into a 100 c.c. Erlenmeyer flask, 0·25 c.c. phenolphthalein solution and 0·25 c.c. of ester are added and the flask brought to 37° C. by gentle heat ; 0·5 c.c. of blood-serum is then added and the flask incubated, without continual shaking, at 37° C. for 2 hours. The flask is then rapidly cooled under the tap, and the contents immediately titrated against N/100 NaOH until a pink tint appears, matching that obtained with the blank control. The control flask contains no serum, but is otherwise similar.

The substrate chiefly used was ethyl butyrate, and in the case of the rat, only the results obtained with this substrate are reported. Tributyrin, phenyl acetate and glyceryl triacetate were also used, but since the general tendency was similar in the rat to that obtained with ethyl butyrate, the results are omitted.

As the experiments were purely comparative in nature, the degree of hydrolysis is expressed as a figure denoting the number of c.c. N/100 NaOH required to neutralize the fatty acids liberated under the standard experimental conditions.

The estimations of the fatty acid and cholesterol content of the serum were carried out by Dr. Jenkinson, and the methods used are described in an appendix to this paper.

CHANGES DUE TO THE JENSEN SARCOMA.

Preliminary experiments indicated that a very large fall in the esterase content of the serum almost invariably occurred in the presence of a Jensen

TABLE I.—*Relation between Size of Jensen Sarcoma and the Esterase and Fat Content of Rat Serum.*

Tumour weight. / Rat weight (%).	Time since inoculation (days).	Blood-serum. Ethyl butyrase (c.c. N/100 NaOH).	Fatty acid. (mgm. per 100 c.c.)	Cholesterol. (mgm. per 100 c.c.)
No tumour	Not inoculated	29·0	214	69
,,	,,	22·6	210	90
,,	16	25·5	186	60
,,	71	41·2	118	80
0·5	24	17·8	..	..
1	16	25·3	161	90
2	7	18·4	171	85
3	8	15·3	..	..
5	16	15·7	164	120
6	14	12·6	280	90
9	19	21·6 (*a*)	..	..
11	16	3·7	1240	140
11	16	1·1	..	..
11	16	3·8	871	130
12	24	2·0	1764	240
12	19	8·1	181	97
13	23	7·9	490	86
14	19	9·9	115	80
15	28	17·1 (*b*)	477	190
15	23	9·6	271	90
16	18	2·2	269	80
19	31	5·7	1350	150
19	25	2·1	1519	180
20	31	12·1 (*c*)	108	72
20	24	5·7	722	120
21	24	5·7	598	130
24	24	1·6	..	..
27	14	8·1	537	114
31	23	2·0	332	70
32	19	1·1	324	100
35	23	2·0	140	100
35	16	3·9	..	..
40	19	0·8	246	90

sarcoma. Coincident with this there was usually a rise in the fatty acid content, often obvious to the naked eye owing to the considerable opacity of the serum. In any small group of rats a precise relationship between the extent of these changes and the size of the tumour was not always evident, but in a survey of many groups it was observed that during the growth of the Jensen sarcoma there was a progressive fall in the esterase content of the serum until it reached almost zero, and a progressive rise in the fatty acid content of the serum. This general tendency can be observed in Table I, where the results obtained in the last 29 sera of sarcoma-bearing rats examined are tabulated according to the relative size of the tumour. It will be seen that there are several apparent exceptions to the general rule, and these are discussed later.

Whilst the fall in the esterase content of the serum is clearly seen to be roughly proportional to the relative size of the tumour, it is obviously impossible to express this relationship graphically from the data obtained in such a series. No doubt many factors besides the actual size of the tumour, such as individual variations in response to a similar stimulus, the age of the rat, the growth-rate of the animal and the tumour, affect the extent of the serum change. Several attempts to correlate these factors with the serum changes in terms that can be expressed mathematically have failed, probably because the number of variables is too great to allow of simple analysis, or the number of determinations required is above practical limits.

The same considerations apply in somewhat greater degree to the changes observed in the serum fatty acid content. Here the original fat reserve of the rat prior to tumour inoculation is probably an important factor in determining the extent of the changes.

Average Results.

The general tendency of these serum changes is seen much more clearly in the average figures obtained from analyses of the sera of 35 rats without tumours and 85 sarcoma-bearing rats (see Table II). In all groups from which these average figures were obtained the control rats corresponded in age and sex to the rats inoculated with sarcoma tissue.

TABLE II.—*Relation between Weight of Jensen Sarcoma and the Esterase and Fat Content of Rat Serum (Average Results).*

Tumour weight (gm.).	Number of rats.	Blood-serum. Ethyl butyrase (c.c. N/100 NaOH).	Number of rats.	Fatty acid. (mgm. per 100 c.c.)	Cholesterol. (mgm. per 100 c.c.)	Remarks.
No tumour	21	27·8	10	168	75	Not inoculated.
,,	14	34·0	6	159	91	Inoculated—no growth.
1–5	12	19·7	12	236	76	—
5–15	15	8·0	12	560	122	—
15–30	38	5·0	34	833	132	—
>30	17	4·1	15	377	105	—

It will be observed that there was a definite fall in the esterase content of the serum even at a very early stage of tumour growth, and with increasing size of the tumour the contrast between the tumour-bearing and the normal

control rats is very striking. Many instances of the almost negligible amount of esterase in the serum of rats with large tumours are included in Tables I and III.

The average esterase content of the serum of inoculated rats in which a tumour failed to grow was definitely higher (34·0) than that of the non-inoculated controls (27·8). In individual members of the "non-take" group a definite increase was noted in only half the cases; 4 of the 14 rats in this group gave figures ranging from 40–45 and 3 figures from 35–40, whilst the remainder were within normal limits. Very occasionally much higher values were obtained in tumour rats than was to be expected from the size of the tumour. It was usually found that the anomaly was associated with either a slow-growing tumour or one which was actually regressing. In Table I three such instances are noted; in two [(*b*) and (*c*)] the tumours were growing very slowly, and in one [(*a*)] the tumour was regressing.

The average fatty acid content of the serum was greater at any stage of tumour growth than in the normal rat. In many cases obvious lipæmia was present when the tumour reached a large size (roughly 10–20 per cent. of the animal's weight), and, as will be seen in Tables I and III, the fatty acid content may rise to nearly 2 per cent. The stage at which the maximum rise in the fatty acids is reached probably varies considerably in individual animals, and it is therefore difficult to be certain whether this rise occurs in all cases, for it was not found possible to carry out several determinations on the same rat at progressive stages of tumour growth. Several instances will be observed where at a fairly early stage of tumour growth the fatty acid content of the serum was around or below normal, and in these cases it cannot be said as yet whether there has been no change in the serum fats, whether a rise would have occurred later, or whether a rise followed by a fall has already occurred. It is obvious from the average results given in Table II that when the tumour has reached a large size the fat content of the serum commences to fall, and in some cases a fall to well below normal was observed. It was noted that these very low values were always obtained in rats showing evidence of profound toxæmia and inanition, and in which the fat reserves were probably completely exhausted. For this reason very great differences were noted in the serum fat contents of rats with relatively enormous growths. In the group of rats bearing tumours weighing more than 30 gm. (see Table II), 8 of the 14 showed a serum fatty acid content of over 400 mgm. per 100 c.c., with an average value of 495, whilst 7 were below 400, with an average value of 244. In the former the average serum esterase value was 5·1, and in the latter 3·3.

In comparison with the fatty acid the cholesterol content of the serum rose relatively slightly, reaching at its maximum an average figure nearly twice the normal value. In individual instances the rise reached a level nearly three times the normal value, but the change was very variable in extent, and often a large increase in the fatty acid was accompanied by no, or only a slight increase in the cholesterol (see Table III).

Relation between Fatty Acid and Esterase Content of Serum.

Since the occurrence of lipæmia has been attributed to a decrease in the amount of blood lipase (Sakai, 1914), whilst Fanconi (1924) states that the

decrease in blood lipase observed in lipæmia is due to adsorption on the fat, it was interesting to see if any relation between these two factors was apparent in the present data.

TABLE III.—*Comparison between Esterase and Fat Content of Serum in the Rat Jensen Sarcoma.*

Fatty acid. (Mgm. per 100 c.c.)	Cholesterol. (Mgm. per 100 c.c.)	Ethyl butyrase (c.c. N/100 NaOH).
45	12	0·4
46	86	25·4
149	70	0·9
161	75	1·5
164	102	4·8
167	128	13·1
176	85	15·3
213	31	22·3
274	124	4·0
314	100	7·2
335	90	15·8
372	120	3·5
394	106	4·7
404	121	9·3
447	90	10·7
456	102	5·4
514	128	4·1
526	112	10·0
596	120	3·1
664	120	3·1
716	84	8·8
888	120	11·7
911	90	5·7
952	121	4·4
1086	163	6·3
1160	140	3·9
1192	210	7·2
1235	190	2·0
1519	180	2·1

Note.—Figures which roughly duplicate any line in this table and those already given in Table I are omitted.

In Table III the serum fatty acid contents observed in a series of sarcoma-bearing rats have been arranged in ascending order of value, with the corresponding values for cholesterol and serum esterase. It will be apparent that though high fatty acid values are generally associated with low esterase values, there is obviously no exact relationship between the two. Moreover

there is no evidence of the converse relationship, for very low esterase values are frequently observed when the fatty acid content of the serum is within normal limits. Additional evidence that these two factors are not directly related was obtained in tar-injected rabbits (*v. supra*), for a high fat content of the serum was noted where the serum esterase value was above normal.

Esterase Content of the Tissues.

It seemed important to determine whether the fall in serum esterase was an isolated phenomenon, or part of a general fall in the esterases of the body tissues generally.

The esterase content of the tissue was determined by removing the tissue with aseptic precautions, grinding a weighed amount with silver sand and a measured quantity of normal saline and filtering through paper (Whatman No. 1). The filtrate was used as a source of tissue esterase.

The average results from individual determinations on the serum of 8 rats with large sarcomas and 8 non-inoculated rats are given in Table IV.

TABLE IV.—*The Esterase Content of the Tissues in Sarcoma-bearing and Normal Rats* (*Average Results* 8 *Rats in each Group*).

Tissue.	Ethyl butyrase (c.c. N/100 NaOH).	
	Cancer group.	Normal group.
Blood-serum	4·4	18·1
Liver	20·6	56·4
Kidney	20·4	30·9
Lung	6·2	22·5
Sarcoma tissue	0·7	..

1 c.c tissue extract (equivalent to 0·1 gm. moist tissue) was used.

There was a very definite reduction in the amount of tissue esterase in all the tumour rats examined. It will be noted that in the case of the liver and lung the reduction was large, whilst in the kidney the fall, though definite, was not so pronounced. The very low esterase content of sarcoma tissue itself should be noted. The individual results showed a striking relationship between the amounts of serum and liver esterase, for in order of magnitude, both followed a parallel course without exception. The average serum esterase value of the control rats was below normal, due to the fact that two of the group had lung infections. Infection has been shown to reduce the esterase content of the serum (Green, 1934), and it was therefore interesting to observe that the tissue esterase content was in these two instances much below that of the healthy rats. The above findings make it highly probable that the serum esterase changes in the rat sarcoma are part of a general tissue change, in which the liver, owing to its size and richness in this enzyme, plays a large part.

Phosphatase Content of the Serum in Jensen Sarcoma.

In order to assess the degree of specificity in the serum esterase change, the effect of the Jensen sarcoma on another serum enzyme was investigated. For this purpose the phosphatase content of the serum was determined.

The technique evolved by Jenner and Kay (1932) was followed in detail, except that the incubation period was 2 instead of 3 hours, and the incubation temperature was 37° instead of 38° C.

Individual determinations of the phosphatase and esterase content of the serum were made in 18 rats bearing large Jensen sarcomata (ranging from 13–48 gm. in weight) and in 14 normal rats. Rather wide variations were found in the serum phosphatase content of both tumour and normal rats, which were probably due in some degree to variations in age. Since, of course, the estimations of the two enzymes were carried out on samples of serum from the same rat, and since the control rat or rats in any one experiment were of the same sex and roughly similar age as the tumour rats, it was considered accurate enough for the purpose in hand to average the results obtained.

TABLE V.—*Comparison between Esterase amd Phosphatase Content of Serum in Normal and Sarcoma-bearing Rats (Average Results).*

	Number of rats.	Units* of phosphatase.	Ethyl butyrase (c.c. N/100 NaOH).
Normal	14	15·3	28·5
Sarcoma	18	9·7	3·8

* Number of milligrammes of phosphorus liberated by 100 c.c. of serum.

It will be seen (Table V) that the phosphatase content of the serum of the tumour rats was distinctly lower than normal, but the reduction from normal is only 37 per cent., as compared with a reduction in the esterase content of 87 per cent. In two cases very low values for phosphatase (3·1 and 1·5 units) were found in tumour rats. These rats had very large tumours, and it is probable that they were nearing the point of death. In many cases the serum phosphatase in the tumour rats only showed a slight reduction from normal, and it would appear from these relatively few results that the presence of a large Jensen sarcoma has not such a profound effect on the production or utilization of this enzyme as it has on the fatty esterases.

THE SERUM ESTERASE CONTENT IN TARRED ANIMALS.

It was obviously desirable to find whether similar changes in the esterase content of the serum to that found in the sarcoma-bearing rat occurred in animals with other forms of tumour and in response to the application of carcinogenic agents. To this end a large number of experiments have been carried out, but the results have been by no means so uniform as those obtained in the rat. The investigation has therefore proved a lengthier task than was expected, and it is proposed at this stage to give little more than a summary of the trend of the findings.

The serum esterase content of mice with tar epitheliomata has in most cases shown no very significant variation from the normal. Where the mice were vigorous and healthy in appearance the average values were slightly above normal, and in one experiment on a group of 12 tumour-bearing mice the serum esterase (ethyl butyrase) content was twice that of a control non-tarred group. Where the tumour mice appeared ill, average values slightly below normal were obtained.

Three Angora rabbits which had developed papillomata of the skin after prolonged tarring showed very high serum esterase values as compared with 3 non-tarred Angoras of the same age. Rabbit serum hydrolyses ethyl butyrate much less rapidly than tributyrin, but in this particular group of tarred rabbits the ethyl butyrase content of the serum was roughly 10 times that of the controls, and was slightly greater than the tributyrinase content, which was roughly twice that of the controls. Observations were made at weekly intervals for 6 months after tarring was discontinued, but the serum esterase content in both groups remained at a fairly constant level. The removal of a large papillomata from one rabbit was without effect on the serum esterase level.

As a result of these preliminary findings several groups of rabbits of various breeds have been investigated on similar lines, observations being made from the commencement of tarring. Difficulties were encountered, in that considerable fluctuations occurred in the serum esterase level from one observation to another in some rabbits during a preliminary observation period of 2 months prior to commencing tarring. In no case, however, was a normal value for ethyl butyrase observed which approached that found in the first group of tarred Angoras. One group of 4 rabbits has now been tarred at intervals for 15 months; one died after 3 months' tarring, and the esterase (ethyl butyrase) content of the serum had risen 50 per cent. above the "normal maximum" level; one developed skin papillomata after 5 months' tarring, but these have not progressed, and the serum esterase is now slightly higher than normal; one developed papillomata after 5 months, which recently have shown evidence of malignancy, but the esterase level has remained at a consistently low level throughout; one has developed no growth, and the esterase level has gradually risen to about twice the normal. In another group of 4 Angora rabbits which have now been tarred for 10 months, only one has shown any significant change in the serum esterase level, which is now about 50 per cent. above normal. This rabbit developed small papillomatous growths after 6 months, but another rabbit in the group which developed similar growths has shown no change from its original low serum esterase content.

The experimental evidence at present is very indefinite, and only suggests that in response to skin tarring the serum esterase content rises in a certain proportion of cases, but is unaffected or even falls in some animals.

THE SERUM ESTERASE IN HUMAN CANCER.

Only a few observations have been made in human cancer, but they do not suggest that any significant changes in the serum esterase content occur.

TABLE VI.—*Esterase Content of Serum in Human Cancer (Average Results).*

Type of case.	Number of cases.	Ethyl butyrase. (c.c. N/100 NaOH).
Carcinoma :		
Local ; patients in good general condition	12	14·0
Evidence of metastases or general toxæmia	6	5·7
Non-cancerous :		
Convalescent cases in good general condition	16	10·8
Presence of acute or subacute infection	23	6·1

In these experiments 1 c.c. of serum was used and the incubation time was 18 hours.

The results are summarized in Table VI, and it will be noted that in human cancer (cases of carcinoma of mouth, throat, lung, stomach and intestine), where the general health was good, the serum esterase content was on the average slightly greater than in non-cancerous " normal " persons, whereas in the terminal stages of cancer it was well below normal, reaching the level observed in acute and subacute bacterial disease. These results are abstracted from those of larger investigation which it is hoped to record later.

THE EFFECT OF INOCULATION OF CARCINOGENIC AGENTS.

It was thought possible that the effect produced in some rabbits by skin tarring was due to some other property of the tar distinct from its carcinogenic property. The effect of intraperitoneal injections of from 0·5 to 2 c.c. of tar was therefore determined. In all cases the injection produced a rapid fall in the serum esterase content, followed by a more gradual rise commencing several days after the injection, which in many cases reached a level considerably above that prior to the inoculation. This was followed by a slow return to the normal, or slightly above the normal, level. In a few cases there was only a slight rise after the original fall, and the serum esterase content remained persistently below normal. As the esterase content rose from the lowest level the serum often became opalescent, and in all cases in which estimations were carried out a large increase in the fatty acid content of the serum was found, values varying from 0·4 to 1·2 per cent being obtained.

Similar, but not as big changes were found after intraperitoneal injections of 1 to 3 c.c. of a 0·4 per cent. solution of 1 : 2 : 5 : 6-dibenzanthracene in olive oil into rabbits, whereas control animals injected with olive oil alone were unaffected. The extent and time-relations of these serum changes cannot yet be expressed with any certainty, in spite of considerable work on the subject, for great individual variations have been noted. Investigations on these lines are still in progress.

THE LIPASE ACTIVATING POWER OF THE SERUM IN EXPERIMENTAL CANCER.

As it seemed possible that serum esterase might be related to the factor in serum which activates pancreatic prolipase, the lipase-activating power of the serum was determined in a few sarcoma-bearing rats, and in rabbits in which the serum esterase content was above normal. The technique described by

Corran and Lewis (1928) was used. No significant changes were observed in the lipase-activating power of the sera of these animals, and in view of the definite changes observed in the serum esterase content, it would appear unlikely that this enzyme is related to the factor acting as co-enzyme to inactive pancreatic lipase.

CONCLUSION.

The significance of the large fall in the serum esterase content which occurs during the growth of the rat Jensen sarcoma is obscured by the fact that other apparently unrelated conditions produce a similar, though probably not so profound a change. In the rat it was shown (Green, 1934) that vitamin A deficiency and infection have a similar effect, and it is possible that toxic agents in general act similarly. The action of the growing sarcoma may be a general depressant action on tissue metabolism. There are one or two facts, however, which suggest that the change may be related more specifically to tumour growth. The change begins at an early stage of tumour growth, when the animal appears vigorous and healthy ; in animals resistant to tumour inoculation the serum esterase content tends to rise ; the phosphatase is not affected to the same extent as the esterase content of the serum, whereas a general toxic action of the sarcoma would probably diminish the production of all enzymes to the same degree.

Some observers have suggested a relationship between the appearance of immune bodies in the blood and an increase in the serum lipase, but the evidence is not convincing. The evidence is more clear that though blood complement and esterase are not identical substances, they show a quantitative relationship (Jobling and Bull, 1913; Gordon and Wormall, 1929). The possibility, therefore, that the serum changes under discussion are related to immunological changes in cancer serum cannot be rejected.

The considerable degree of anæmia which occurs during rapid tumour growth with a resultant increase in the plasma content of the blood may be a partial cause of the fall in the serum esterase. The extent of the fall, and the fact that the phosphatase is not affected to the same extent, indicate that this is not the sole cause. It is also possible that the rise in the fatty acid content of the blood is associated with anæmia. Boggs and Morris (1909) and Horiuchi (1920) showed that repeated bleedings in rabbits produced a marked rise in the blood fat, though an anæmia produced by a toxic agent (pyridine), which is more analogous to the present case, produced no change in the blood fats. The fact that the blood fat tends to rise after the injection of carcinogenic agents into rabbits suggests, however, that there may be a more specific relation between tumour growth and the mobilization of fat into the blood.

Nothing further can be added as to the cause of the lipæmia observed in these experiments, for the cause of such well-known instances of lipæmia as occur in diabetes, anæmia and phlorrhizin poisoning is not known. A consideration of the known facts of fat mobilization (see Leathes and Raper, 1925) renders it improbable that it is due to a large demand for fat in tumour metabolism. The possibility that the changes in the fat and esterase of the serum may be an expression of some abnormality of fat metabolism in tumour tissue

must, however, be considered. Experiments are in progress to determine whether tumour extracts will bring about similar changes when injected into the normal animal.

Many activating substances (*e.g.* calcium, albumen, bile salts) for pancreatic lipase are known, and it is probable that there are similar activating factors for the tissue esterases. The tumour-cell may conceivably produce some substance having an inhibitory effect on esterase action, or it may destroy some activating substance in the body tissues. Experiments with tumour extracts *in vitro* have not up to now revealed the presence of an inhibiting agent.

The fact that a fall in the esterase content of the serum is not found in animals with tar epitheliomata, and that there is a tendency for the reverse effect to occur, is possibly due to the enormous difference in the rate of growth of these tumours compared with that of the rat sarcoma. It is tempting to suggest that this change is an expression of the resistance of the animal to tumour growth. With a rapidly growing tumour the esterases of the body tissues rapidly decrease in amount, whilst in an animal resistant to tumour inoculation the esterases tend to increase. In a slow-growing tumour there is only a slight alteration to above or below the normal according to the degree of resistance set up by the tissues. In response to a large dose of a carcinogenic agent there is a preliminary fall due to the normal resistance being temporarily overcome, followed by a rise to above the normal level as the resistance of the animal increases. This is, of course, a purely hypothetical explanation, but it may serve to link up the apparently disconnected facts until more is known. A truly adequate explanation of these experimental facts may not at present be possible, for we are dealing with factors of which our physiological knowledge is notoriously lacking.

SUMMARY.

1. During the growth of the rat Jensen sarcoma the esterase content of the serum falls progressively, ultimately reaching a very low level. The esterase content of the liver, lung and kidney is also much diminished.

2. The phosphatase content of the serum also falls, but the average fall is less than half that of the esterase.

3. In rats resistant to inoculation of the Jensen sarcoma the esterase content of the serum tends to rise.

4. The fatty acid content of the serum rises in many, and possibly all rats during the growth of the Jensen sarcoma. It may reach a maximum level, approaching 2 per cent., and then falls during the terminal stages of tumour growth. There is an associated rise in the cholesterol, but of a much less degree.

5. In tar epitheliomata of mice and in localized human carcinomata the serum esterase content ranges around normal, with a tendency to rise slightly.

6. Evidence was obtained that the application of tar or the inoculation of tar or 1 : 2 : 5 : 6-dibenzanthracene produces a rise in the esterase content of the serum of rabbits in a proportion of cases.

7. The possible significance of these findings is discussed.

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APPENDIX.

THE ESTIMATION OF FAT IN BLOOD-SERUM.

C. N. JENKINSON.

From the Department of Physiology, University of Sheffield.

As it was found that the amount of fat in serum from defibrinated or clotted blood was the same as that in plasma from blood collected with oxalate, serum was used in this work for determining the degree of lipæmia.

The method used was that described by Leathes and Raper (1925), adapted to much smaller quantities than were contemplated there. The serum, generally 1 c.c., is measured into a small round, long-necked, stoppered flask ; the capacity of the bulb is about 30 c.c., that of the neck about 20 c.c. An equal volume of strong caustic potash solution is introduced, and the mixture heated in a boiling water bath for a few minutes, when, the coagulum having dissolved, 5 c.c. of alcohol is added and the heating continued for an hour. The flask is then cooled, the contents acidified with 40 per cent. sulphuric acid, and after again cooling, diluted with water so as to fill the flask to the bottom of the neck. Exactly 15 c.c. of light petroleum (B.P. 40°–50° C.) is run in. This leaves an air-space beneath the stopper sufficient to allow satisfactory shaking. The flask is then shaken by hand six or seven times at intervals of a minute or two, each time for 30 seconds, and then left to allow the liquids to separate. Then 10 c.c. of the petroleum solution is transferred to a small round weighed flask, which weighs about 6–7 gm., and has a capacity of 12–15 c.c. The neck is S-shaped, so that it can be passed through a cork in the wide end of a Liebig condenser for removal of the solvent. This is distilled from a warm water bath, the last traces being removed by connecting the receiver with a vacuum water pump for about 15 minutes. After standing in a desiccator for some hours the flask and residue is weighed.

The material weighed contains besides the fatty acids released by saponification of glycerides and lipins, unsaponifiable matter, mainly cholesterol. This latter is estimated colorimetrically. Five c.c. of chloroform is run into the flask ; 2 c.c. acetic anhydride and 0·1 c.c. concentrated sulphuric acid are added ; at the same time 5 c.c. of a standard solution of cholesterol containing 0·4 mgm. is treated similarly, and after standing in the dark for 10 minutes the colour developed in the two solutions is compared.

Using this method the average difference between duplicate analyses for fatty acids and their mean was just $\pm$ 2 per cent.

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STUDIES ON PROTEIN-FREE SUSPENSIONS OF VIRUSES. V: FURTHER OBSERVATIONS ON THE NATURE AND ANTIGENIC PROPERTIES OF A HIGHLY PURIFIED PHAGE.

I. J. KLIGLER AND L. OLITZKI.

From the Department of Hygiene and Bacteriology, Hebrew University, Jerusalem.

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IN a previous publication (Kligler and Olitzki, 1931) it was reported that by adsorption with kaolin and elution with ammonia it was possible to obtain protein-free suspensions of a bacteriophage and virus. It was also shown that the purified virus and phage reacted differently to heat, disinfectants, etc., than they did in the presence of proteins. Recently Rakieten and Hunt (1932) claimed to have been unable to confirm our findings.

Furthermore we found that although the purified phage suspensions gave negative tests for protein (Esbach sulfosalicylic acid), they gave a positive ninhydrin reaction and contained fairly high amounts of non-ammonia nitrogen (9–11 mgm. per 100 c.c. solution). It was considered desirable, therefore, to standardize the technique and to attempt further purification of the eluate. The present paper deals with the elution and purification of a coli and typhoid phage, and a study of the antigenic properties of a highly purified phage.

STANDARDIZATION OF ADSORPTION AND ELUTION TECHNIQUE.

The first problem was to standardize the technique in order to insure uniform results. As in all processes involving the use of reagents having a variable character—kaolin, culture media, etc.—the method used and the conditions under which the experiments are made exert a considerable influence on the results.

Media and reaction.

The character of the medium and the reaction at which adsorption takes place influence the amount of phage produced, the degree of adsorption and the readiness with which the phage is subsequently eluted. Beef extract or beef infusion broth are favourable for these purposes, whereas in synthetic medium the yield of phage is low and the subsequent elution is unsatisfactory. The following protocols are illustrative :

Parallel tubes of beef extract broth and of a synthetic medium (*) were seeded at the same time with the same amounts of culture and phage, incubated at 37° C., and then filtered through Seitz filters. The phage strength of the broth was 10^{-8}, and of the synthetic medium 10^{-5} per c.c.

The adsorption and elution results with the two phages were as follows :

	Phage titre in—		
	Supernatant fluid.	Kaolin sediment.	Eluate.
Broth :			
Adsorbed at : pH 5·5 . . .	10^{-2}	10^{-6}	10^{-8}
pH 7·6 . . .	10^{-3}	10^{-9}	10^{-7}
Synthetic medium :			
Adsorbed at : pH 5·5 . . .	0	10^{-3}	10^{-2}
pH 7·6 . . .	10^{-1}	10^{-4}	10^{-1}

It is evident that in the synthetic medium the adsorption is of such a nature that elution is slight in comparison with that obtained in the broth medium. It is also apparent that a better eluate results when the adsorption is carried out in an acid medium (pH 5·5) than when it is done in an alkaline medium. Repeated experiments gave consistent results.

Adsorbent.

Kaolin proved to be the most satisfactory adsorbent. Repeated experiments indicated that 25 per cent. kaolin gave best results. The kaolin is prepared by autoclaving 1·0 gm. in 2 c.c. saline containing 0·4 c.c. phosphate buffer of pH 5·5. To this sterile suspension are added 2 c.c. of the phage. The material is shaken thoroughly and left overnight at ice-box temperature.

The following experiment is typical :

A broth phage was divided into four parts; one part served as control; the three other portions were adsorbed with buffered kaolin suspensions having different reactions. The same amount of adsorbent was used in every case. The results were as follows :

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* The synthetic medium was made up as follows :

Glycocol . . .	0·5 gm.	$CaCO_3$	0·3 gm.
Cystin	0·05 ,,	KH_2PO_4	0·1 ,,
Glucose . . .	0·5 ,,	$MgSO_4$	0·1 ,,
Water	100·0 c.c.	$(N_3)H_2SO_4$ . . .	0·2 ,,

Reaction of adsorbent-pH.	Titre of phage.		
	Supernatant fluid.	Kaolin sediment.	Eluate.
5·5	10^{-3}	10^{-5}	10^{-9}
6·8	10^{-3}	10^{-7}	10^{-8}
7·6	10^{-3}	10^{-7}	10^{-8}
Control (unbuffered)	10^{-3}	10^{-5}	10^{-8}

Original phage titre 10^{-9}.

Adsorption with 25 per cent. kaolin at pH 5·5 yielded on elution a phage suspension having the same titre as the original broth, while at other reactions the yield was lower.

Kaolin is a variable substance, and consequently each lot should be tested to ascertain the best concentration to be used for adsorption, so as to yield an eluate of maximum strength.

Purification of Eluate.

The major objective of these experiments was to ascertain whether it is possible to obtain a phage suspension of high titre entirely free, or nearly so, of foreign substances. To this end various methods were used.

The first procedure was to readsorb the eluate with varying amounts of kaolin or aluminium hydroxide. Amounts equivalent to 5, 2, 1 and 0·5 per cent. were added to aliquot portions of eluate, shaken for 1 hour at room temperature, then centifuged, and the phage titre and N content of the clear fluid determined. It was found that readsorption with kaolin removed large amounts of phage, the amount removed varying directly with the amounts of kaolin and with the acidity of the suspension. Aluminium hydroxide, on the other hand, removed the foreign substance with only a moderate reduction in the phage strength.

Experiment 1.—In this experiment an eluate having a phage strength of 10^{-9} was shaken one hour with 2·5 per cent. suspension of $Al(OH)_3$ (pH 7·0), and centrifuged. The clear fluid was then dialyzed for five days and again treated with 8 per cent. $Al(OH)_3$. The results were as follows :

	Titre.	Protein test.	Ninhydrin.	Non-ammonia N.
Original eluate	10^{-9}	—	+	9·80 mgm. per cent.
After treatment with $Al(OH)_3$ (2·5 per cent.)	10^{-10}	—	+	9·24 ,, ,,
After dialysis (5 days)	10^{-9}	—	—	8·12 ,, ,,
After second treatment with $Al(OH)_3$ (8·0 per cent.)	10^{-7}	—	—	5·88 ,, ,,

Experiment 2.—In this experiment the treatment with $Al(OH)_3$ was eliminated, and only dialysis used. The phage strength of the eluate was 10^{-8} and N. content 7·00 mgm. per cent.

After dialysing 48 hours phage = 10^{-8} . N. = 6·44 mgm. per cent.
,, ,, 120 ,, ,, 10^{-8} . N. = 4·50 ,, ,,

Experiment 3.—In this experiment the eluate obtained from a kaolin adsorbate was readsorbed with kaolin and then eluted in the usual manner.

The phage strength of the first eluate was 10^{-8}, and the N. content 11·48 mgm. per cent. Esbach and sulfosalicylic acid tests were negative; the ninhydrin test was positive. 4 c.c. of this eluate was again treated with 2 gm. kaolin and 0·5 c.c. N/15 phosphate buffer (pH 5·5) for 24 hours. The sediment was then treated with 8 c.c. N./100 $NH_4(OH)$.

The supernatant fluid after the readsorption had a phage strength of 10^{-5}, an N. content of 6·90 mgm. per cent., and slightly positive ninhydrin test. The last eluate had a phage strength of 10^{-7}, an N. content of 3·64 mgm. per cent. and a slightly positive ninhydrin reaction. The eluate was then dialyzed against distilled water for 6 days. After dialysis the ninhydrin test was negative, the phage strength was 10^{-6}, and the N. content 1·4 mgm. per cent.

Experiment 4.—The former experiment was repeated with some modifications. Instead of repeated readsorption and elution, repeated elutions were made from the same adsorbate. The elutions were made with and without previous washing of the kaolin sediment with saline. The second eluate always had a lower N. content, but the ninhydrin reaction remained positive until after dialysis. The following is a typical protocol :

	Phage strength.	N. content.
Original phage	10^{-9} .	3·15 mgm. per cent.
Saline washings	10^{-3} .	7·98 ,, ,,
Unwashed sediment—1st eluate .	10^{-9} .	8·52 ,, ,,
,, ,, 2nd ,, . .	10^{-8} .	4·48 ,, ,,
Washed sediment—1st eluate . .	10^{-8} .	6·16 ,, ,,
,, ,, 2nd ,, . .	10^{-7} .	4·20 ,, ,,

The dialysis, if carried long enough, removed the ninhydrin reacting amino-acids and further reduced the N. content. The following is an example :

	Phage strength.	N. content.
2nd eluate before dialysis . . .	10^{-9} .	4·2 mgm. per cent.
,, ,, after dialysis (48 hours) .	10^{-9} .	3·9 ,, ,,
3rd ,, before dialysis . . .	10^{-8} .	2·2 ,, ,,
,, ,, after dialysis (48 hours) .	10^{-8} .	1·9 ,, ,,

These experiments showed clearly that it is possible by repeated elution from the same adsorbate followed by subsequent dialysis to obtain potent phage suspensions, giving negative protein and ninhydrin tests, and having an N. content (non-ammonia) of only 1·4 to 2·5 mgm. per cent.

Total Phage Strength.

An interesting point brought out by these experiments was that repeated elution of a given adsorbate continued to give potent phage suspensions, and that the total phage titre of the serial eluates was at times higher than that of the original broth phage. This peculiar phenomenon is clearly brought out by the following protocols :

	Phage titre: broth dilutions.							
	Typhoid phage.				Coli phage.			
		Esbach.	Ninhydrin.	N. (mgm.%).		Esbach.	Ninhydrin.	N. (mgm.%).
Original phage	10^{-10}	..	..	..	10^{-11}	..	..	..
Supernatant fluid	10^{-3}	..	..	..	10^{-4}	..	..	..
After adsorption:								
Eluate 1	10^{-8}	—	++	18·06	10^{-10}	—	++	10·08
,, 2	10^{-8}	—	±	5·32	10^{-8}	—	±	4·28
,, 3	10^{-7}	—	Trace	3·08	10^{-8}	—	Trace	2·24
,, 4	10^{-3}	—	,,	3·08	10^{-4}	—	—	1·40

This experiment was repeated, using both broth and plate method for estimating the phage strength. The results were essentially the same, but the agar method brought out the results more sharply.

	Coli.		Typhoid.	
	Broth dilution.	Agar plaques.*	Broth dilution.	Agar plaques.*
Original phage	10^{-9}	3·8	10^{-10}	12·70
Supernatant	10^{-4}	0·5	10^{-2}	0·14
Eluate 1	10^{-8}	6·4	10^{-9}	12·00
,, 2	10^{-7}	3·6	10^{-7}	7·20
,, 3	10^{-5}	0·6	10^{-7}	6·40
,, 4	10^{-1}	0·0	10^{-2}	0·25

* Million plaques per c.c. of phage or eluate.

These experiments suggested the possibility that part of the phage present in the original culture was inactivated by the bacterial products. If that were the case, then it would be expected that the richer the culture the greater the inactivation. To test this possibility phage was prepared under identical conditions, except that one set of tubes were incubated at 37° C. and the other at 20° C. The results are tabulated below:

(A) *Adsorption and Elution at 20° C.*

	Coli phage.		Typhoid phage.	
	Prepared at 37° C.	Prepared at 20° C.	Prepared at 37° C.	Prepared at 20° C.
Original	10^{-9}	10^{-8}	10^{-9}	10^{-8}
Kaolin adsorbate	10^{-8}	10^{-7}	10^{-6}	10^{-4}
Supernatant fluid	10^{-2}	10^{-3}	10^{-4}	10^{-3}
Eluate 1	10^{-9}	10^{-6}	10^{-7}	10^{-4}
,, 2	10^{-8}	10^{-3}	10^{-7}	10^{-1}
,, 3	10^{-6}	0	10^{-6}	0
(B) *Adsorption and Elution at 37° C.*				
Original	10^{-9}	10^{-8}	10^{-9}	10^{-8}
Kaolin adsorbate	10^{-9}	10^{-8}	10^{-8}	10^{-7}
Supernatant fluid	10^{-2}	10^{-1}	10^{-1}	10^{-1}
Eluate 1	10^{-7}	10^{-5}	10^{-6}	10^{-3}
,, 2	10^{-5}	10^{-2}	10^{-4}	10^{-1}
,, 3	10^{-2}	10	10^{-1}	0

These experiments indicate that although the broth titre is about the same, more phage is produced at 37° C, than at 20° C., and that at least part of the phage produced at 37° C. is inactivated. They show further that adsorption and elution at 37° C. leads to a considerable destruction of the phage, confirming an observation recently reported by Dreyer and Campbell-Renton (1933).

INHIBITING OR INACTIVATING SUBSTANCE.

The above experiments indicated that there was some inhibiting substance in the broth phage which was apparently removed by adsorption. We therefore extracted the residual material which remained on the kaolin after repeated elutions, and tested its effect on phage. A large amount of phage was adsorbed and eluted in the usual way. After the fourth elution the kaolin residue was treated with N/10 NaOH, thoroughly shaken, and left at 37° C. for 2 days. The suspension was centrifuged, the clear supernatant fluid removed, neutralized with N/10 HCl and filtered.

Equal amounts of this extract were added to beef extract broth, and the mixture used for titrating the strength of a coli phage. The test was set up as follows :

Coli-Phage Titration

(In broth and protein extract from kaolin adsorbate.)

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
(1) 2·5 c.c. bouillon plus 2·5 c.c. NaCl.	—	—	—	—	—	—	++	++	+++	+++
(2) 2·5 ,, bouillon plus 2·5 c.c. HCl-NaOH (neutral)	—	—	—	—	—	—	—	+	+++	+++
(3) 2·5 ,, bouillon plus 2·5 c.c. coli extract	+	+	+	++	++	++	++	+++	+++	+++
(4) 2·5 ,, bouillon plus 2·5 c.c. typhoid extract	+	++	++	+++	+++	+++	+++	+++	+++	+++
(5) 2·5 ,, bouillon plus 2·5 c.c. extract from broth*	—	—	—	—	±	+	+	++	++	+++

— = Complete lysis ; ± = slight growth ; +, ++, +++ = well-marked growth of different densities.

* This extract was obtained by treating sterile broth in the same manner as the phage, and served as a control of the specific or non-specific character of the inhibitive substance.

The phage-inhibiting effect of the alkaline extract obtained from the kaolin adsorbate was demonstrated by another type of experiment. A parallel series of tubes was set up, one containing equal parts of broth and extract, the other broth alone. The same amount of a coli culture and serial dilutions of a coli phage were added to the parallel sets. The series containing extract showed marked inhibition. The tubes of dilution 10^{-4} were then filtered through Seitz filters, and the phage strength of each determined. The results are shown in the protocol :

	Phage titre.				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
(*a*) Tubes without extract	—	—	—	—	—
(*b*) Tubes with extract	—	±	+	+	++

The tubes of 10^{-4} were filtered and titrated :

	Phage titre.				
	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}
Tube a^4	—	—	—	—	+++
Tube b^4	±	±	+	++	+++

On plates—Tube a^4 gave in 10^{-2}, ∞ plaques in 0·1 c.c.
10^{-4}, 640 ,, 0·1 ,,
Tube b^4 ,, 10^{-2}, 1280 ,, 0·1 ,,
10^{-4}, 200 ,, 0·1 ,,

It is apparent that there are substances in coli and typhoid phage cultures which have a marked inhibitive effect on phage. Similar extracts from sterile broth also yield an inhibitive substance in lesser concentration. The nature of this substance and whether the broth and culture extracts are identical and hence non-specific, or a mixture of specific and non-specific substances, is a subject now being investigated.

ANTIGENIC PROPERTY OF PURIFIED PHAGE.

The extreme purity of the newly obtained phage suspension necessitated the re-testing of its antigenic character. In our previous experiments we were dealing with phage suspensions which gave negative protein tests (Esbach), but still contained relatively large amounts of non-ammonia nitrogen. With the further purification it seemed necessary to ascertain whether the phage in the relatively purer state retained its antigenic identity.

A phage suspension was prepared which gave negative protein and ninhydrin reactions, and had a nitrogen content of 1·9 mg. per 100 c.c. of solution. This material was used for immunizing rabbits. The rabbits were given five intravenous injections of 1·0, 1·5, 2·0, 2·0, and 2·0 c.c. of the phage suspension at intervals of 3 days. Six animals were immunized in the following manner :

1. Rabbit 305 received phage alone.
2. ,, 304 ,, ,, in 1 per cent. aluminium hydroxide.
3. ,, 309 ,, ,, in 2 ,, gelatin.
4. ,, 314 ,, ,, in 2 ,, gelatin and 1 per cent. aluminium hydroxide solution.
5. ,, 307 ,, ,, in goat-serum (heated to 70° C.).
6. ,, 311 ,, ,, in ,, plus 2 per cent. aluminium hydroxide.

The animals were bled 10 days after the last injection and the sera tested. The neutralizing power of the sera was tested by mixing equal parts of a 1 : 10 dilution of the serum with 1 : 1000 and 1 : 10,000 dilutions of the phage respectively, and incubating 2 hours at 37° C. and then overnight at 20° C. The neutralizing effect of the antiserum was tested by the agar plaque method. Original sera as well as saline controls were run at the same time. The results are shown in Tables I and II.

TABLE I.—*Neutralizing Effect of Antisera Produced with Pure Phage.*

After incubation of 2 hours at 37° C., and 24 hours at 20° C.

Type of sera.	Phage dilution.	Plaques per plate. Without complement.	With complement (0·2 c.c.)
Normal serum and saline	1/1000	80–150	70–120
Controls	1/10,000	8–25	14–27
Immune serum 314	1/1000	0	0
	1/10,000	0	0
,, ,, 304	1/1000	15	24
	1/10,000	2	6
,, ,, 305	1/1000	22	10
	1/10,000	0	0
,, ,, 307	1/1000	28	33
	1/10,000	4	0
,, ,, 309	1/1000	18	26
	1/10,000	0	2
,, ,, 311	1/1000	37	27
	1/10,000	5	0

TABLE II.—*Neutralizing Effect of Antisera Produced with Pure Phage.*

Serum dilution 1 : 5 ; complement 0·2 c.c. to 1 c.c. of mixture ; phage dilution 1/100, 1/1000 ; incubation 2 hours at 37° C. ; overnight at 20° C.

Sera.	Phage dilution.	Plaques per plate. Without complement.	With complement (0·2 c.c.).
Normal serum 314 before immunization	1/100	∞	∞
	1/1000	720	720
Immune serum 314	1/100	240	120
	1/1000	60	0
Normal serum 305 before immunization	1/100	∞	∞
	1/1000	640	560
Immune serum 305	1/100	360	200
	1/1000	40	3
Saline control	1/100	∞	∞
	1/1000	720	640

The first table gives the results of the comparative tests with the various antisera. All antisera contained phage antibodies, though some, *i. e.* 314 and 304, seemed more potent than others.

Since our chief interest was in the antigenic properties of the pure phage the tests were repeated, comparing the antiserum of rabbit 305 produced with phage alone, with that of 314 the most potent antiserum of the series.

The results are shown in Table II. This test confirms the previous one. Both antisera possess phage-neutralizing properties, serum 314 being apparently somewhat more potent than serum 305.

DISCUSSION.

The experiments presented above bring out a number of interesting points bearing on the nature of phage. It is clear that by means of successive elutions of the kaolin adsorbate and subsequent dialysis, it is possible to obtain a potent phage suspension free from extraneous substances. In one instance a purified phage suspension had 10^{-11} particles of phage per 100 c.c., and contained only 1·4 mgm. N. It would seem, therefore, that the material approximates to a pure suspension of phage elements.

Of considerable interest is the fact that by repeated elution of the same kaolin sediment one obtains a larger amount of phage than is apparently contained in the original material. Repeated extraction of the kaolin adsorbate continued to give active phage, each extraction containing less N. than the previous one. In this respect the results resemble those reported by Murphy (1932) in his studies of chicken tumours.

Murphy has further demonstrated that in the chicken tumour there is an inhibiting principle in the cells which masks or partly neutralizes the active agent. Levine (1933) has shown that extracts of normal bacteria prepared in a certain way inactivate or bind phage. In preliminary experiments we have been able to confirm these findings. It appears that the products of the phage-lysed cells exert a phage-inhibiting effect, since alkaline extracts of the residual kaolin adsorbate after repeated elutions proved to have a marked inhibitive effect on the phage. These extracts give a strong protein reaction, and when added to broth markedly inhibit the action of the phage. There is, therefore, a close parallel between our results with phage and Murphy's findings with the chicken tumour virus.

Finally, it is interesting to note that a highly purified potent phage suspension (10^{-9} c.c.), which gives negative protein and ninhydrin tests, and contains only 1·9 mgm. of N. per 100 c.c. of suspension, is still actively antigenic. A total of 8·5 c.c. of this material or about 8·5 billion phage elements, given in five injections, produced an active phage-neutralizing serum in rabbits. In this respect also the phage resembles the purified agent of chicken tumours. The nitrogen content of our purified phage is about 1·5 to 2 times that reported by Murphy in his purified tumour extracts.

CONCLUSIONS.

1. Kaolin adsorption of phage from broth cultures followed by elution with N/100 NH_4OH yields a potent phage relatively free from protein.

2. Successive elutions from the same kaolin adsorbate followed by dialysis yield active phage suspensions giving negative protein and ninhydrin reactions.

3. The residue on the kaolin after repeated elutions has a marked inhibitive effect on the phage. This effect does not appear to be specific.

4. The purified phage retains its antigenic potency.

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OPTIMAL AGGLUTINATION: THE SIGNIFICANCE OF THE DIFFERENT RATIOS OF SERUM TO BACTERIA.

J. T. DUNCAN.

From the Division of Bacteriology and Immunology, London School of Hygiene and Tropical Medicine.

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THE SIGNIFICANT SERUM-SUSPENSION RATIOS.

IN taking comparative readings of agglutination reactions, three different criteria may be used:

(*a*) The position of optimal, that is most rapidly progressive, agglutination when a series of falling doses of agglutinating serum, commencing with a relatively strong concentration, is titrated against a fixed dose of a light bacterial suspension (da Costa Cruz, 1929; Bier, 1931; Duncan, 1932; Miles, 1933). This test is analogous to the Ramon (1922) method of measuring the optimal neutralizing ratio of diphtheria antitoxin to toxin. For the same reacting system, using different concentrations of the bacterial suspension, the ratio of the serum dose to the suspension dose giving optimal agglutination is constant (Duncan, 1932). In the present paper this optimal ratio will be referred to as the "A" *ratio* and the serum/suspension titration arrangement as an "A" *series*.

(*b*) The position of optimal agglutination when a series of falling doses of the bacterial suspension, commencing with a dense suspension, is titrated against a fixed dose of the agglutinating serum (Duncan, 1932; Miles, 1933). This is analogous to the Dean and Webb (1926) method of determining the optimal ratio of serum antigen to antiserum. This optimal ratio will be referred to as the "B" *ratio* and the titration series as a "B" *series*. The "B" optimal ratio, although differing from the "A" optimal ratio for the same reacting system, is constant for all serum dilutions (Duncan, 1932).

(*c*) The end-point or highest serum dilution at which *complete* agglutination occurs, after 24 hours' incubation at a suitable temperature, when the test is arranged in an "A" series. This serum/suspension ratio, which will be

referred to *provisionally* as the " C " ratio, was found constant for all end-point readings with the same reacting system at a salt concentration of M/7. (In the discussion at the end of this paper, " C " ratio is employed in a more restricted sense.) The end-point of complete agglutination has been adopted in preference to an arbitrary end-point, because it gives a constant serum/suspension ratio and its determination presents no difficulty. Arbitrary end-points, which are usually read in the zone of incomplete agglutination, and depend upon such criteria as the ultimate trace of agglutination or the development of a particular size of flocculus under stated conditions of temperature, time, etc., may bear a true relation to the end-point of complete agglutination, but, in practice, the personal element tends to affect the accuracy of reading such arbitrary end-points, especially when suspensions of different densities are involved.

All three ratios, " A ", " B " and " C ", at M/7 salt, bear a constant relation to one another. In the present work it was observed in the agglutination of *Bact. typhosum* (" O " antigen) by *Bact. enteritidis* serum, and in agglutination of *Bact. dysenteriæ* Flexner " Z " by the homologous serum, at M/7 salt, that the " A " ratio showed approximately eight times the serum/suspension proportion of the " B " ratio, and the " B " ratio gave twice the serum/suspension proportion of the " C " ratio. Experience with other bacterial species and sera suggests, however, that the serum relations of the three ratios mentioned are not common to all species.

TABLE I.—*Relations of the " A ", " B " and " C " Serum/Suspension Ratios.* Bact. enteritidis *Serum and* Bact. typhosum *Suspension Approximately* 2×10^{10} *Organisms to* 1·0 *c.c.*

Suspension dilutions.	Serum dilutions.								
	1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640	1/1280.
1/2	..	..	..	..	..	..	..	..	..
1/4	..	..	..	..	C	..	..	..	..
1/8	..	..	..	..	B	C	..	..	..
1/16	..	..	A	..	..	B	C	..	..
1/32	..	..	..	A	..	..	B	C	..
1/64	..	..	..	..	A	..	..	B	..
1/128	..	..	..	..	..	A	..	..	..

Table I shows the relations of the three serum/suspension ratios to one another in the case of the *Bact. enteritidis* serum and *Bact. typhosum* suspension. Ratios " A " and " C " were obtained by transverse readings and ratio " B " by vertical readings. It will be seen that all three ratios are constant and, reading the transverse lines, that they are related to one another in the serum proportions mentioned above.

The same stock of *Bact. enteritidis* serum was tested with 8 strains of normal smooth *Bact. typhosum* from different sources, and in every instance repeated tests showed the relations of the ratios to be those illustrated in Table I. Numerical estimations of the suspensions used showed that the ratios of serum to bacterial mass were approximately similar for all 8 strains.

The reverse experiment—testing several sera with a single suspension—was made with 6 Flexner "Z" dysentery sera and the single culture used for their production. Although the serum/suspension ratios varied with the different sera, the relations of these ratios to one another were approximately similar to those shown in the case of the *Bact. enteritidis* serum and *Bact. typhosum*.

The results of 8 separate experiments, using the same stock of Flexner "Z" serum and 7 different suspensions from the single homologous strain, gave the following approximate ratios of serum to bacteria :

Ratio "A", serum 1·0 c.c. to suspension 1·0 c.c. $= 32 \times 10^9$.
Ratio "B", serum 1·0 c.c. to suspension 1·0 c.c. $= 256 \times 10^9$.
Ratio "C", serum 1·0 c.c. to suspension 1·0 c.c. $= 512 \times 10^9$.

All of the experimental work described hereafter was done with a single Flexner "Z" strain and six different samples of the homologous serum. Its chief purpose is to study the factors influencing the three different serum/suspension ratios with a view to deciding their possible value in quantitative agglutination or absorption tests.

The bacterial suspensions were prepared by washing off 18 hours old agar-plate cultures with M/7 saline, centrifuging and resuspending the deposit in M/7 saline containing 0·3 per cent. formalin ; no heat was employed in sterilizing. The reactions of the reagents in the agglutination tests were between pH 6·8 and pH 7·2, and were thus remote from the iso-electric point of serum globulin. Agglutination tests were incubated at 45° C. or 50° C. for 24 hours to allow completion of the reaction.

ESTIMATION OF THE AMOUNT OF ANTIBODY COMBINED AT THE DIFFERENT SERUM/SUSPENSION RATIOS.

I. *Measurement of Antibody Residues in the Supernatants.*

An "A" series of serum/suspension mixtures was centrifuged after allowing time for antibody-bacteria union, and the supernatants and deposited, sensitized bacteria were examined. The supernatants were carried through a series of dilutions in M/7 saline and tested with a suspension of the homologous bacillus containing 2×10^8 organisms in 1·0 c.c. This density of suspension was used as a standard for end-point tests ; the highest dilution of serum or supernatant liquid causing *complete* agglutination of an equal volume of the suspension was assumed to contain one arbitrary agglutinin dose. The amount of *free* agglutinin in the supernatant measured in this way was accepted as a guide to the amount of agglutinin bound to the bacteria.

Table II gives the results of such an experiment. The central column shows the serum dilutions which were added to an equal volume of a suspension containing 8×10^8 organisms in 1·0 c.c. constituting the initial reaction. The positions of the three ratios "A", "B" and "C", determined by a separate test, are marked. In Part 1 the supernatants from the initial reactions, carried through a series of dilutions, expressed as dilutions of the original serum, show that between the "A" ratio and the highest serum/suspension ratio there is a progressive increase in the amount of antibody

TABLE II.—*Part 1: Titration of Agglutinin Left Free in Mixtures Containing Varying Serum/Suspension Ratios.*

Dilutions of supernatants in terms of original serum.									Serum dilutions absorbed with an equal volume of a suspension of 8×10^8 organisms in 1 c.c.
1/2560.	1/1280.	1/640.	1/320.	1/160.	1/80.	1/40.	1/20.	1/10.	
—	+s.	———	———	———	———	———	———	———	1/5
—	tr.	+	———	———	———	———	———		1/10
—	—	tr.	———	———	———	———	..	..	1/20
—	—	—	+	———	———	..	..	..	1/40 "A"
—	—	—	tr.	———	..	..	..	..	1/80
—	—	—	tr.	..	..	..	..	..	1/160
—	—	—	..	..	..	..	..	..	1/320 "B"
—	—	..	..	..	..	..	..	..	1/640 "C"
—	..	..	..	..	..	..	..	..	1/1280
..	..	..	..	..	..	..	..	..	1/2560

Supernatants titrated against a suspension containing 2×10^8 organisms in 1·0 c.c.

residue, which is relatively greater than the increase in the serum/suspension ratio. It is apparent, therefore, that although the amount of antibody combined increases with the serum/suspension ratio, the combined portion represents a falling percentage of the total antibody.

Assuming that 1·0 c.c. of the serum contained 2560 arbitrary agglutinin doses for 1·0 c.c. of the standard 2×10^8 suspension, or 640 doses for the 8×10^8 suspension, the ratio "A" proportion of serum would contain 64 of the smaller agglutinin doses, of which not less than four remained in the supernatant, which suggests that approximately 93·75 per cent. of the original antibody dose of this ratio has been bound. In a number of experiments, similar to that illustrated, estimations of the amounts of antibody bound gave, approximately, at the "A" ratio 93 per cent. of the total antibody; at twice the "A" serum/suspension ratio, 87 per cent.; at four times, 83 per cent.; at eight times, 75 per cent., and at sixteen times, 67 per cent.

Presumably a stage of antibody saturation of the organisms can be reached, but, in the present work, using the lightest practicable suspension with serum at 1 in 2, this point, apparently, was not attained. It is probable that it could be reached with a more potent serum.

With serum/suspension ratios less than the "A" ratio, the diminishing residues of antibody in the supernatant soon become too small for even

TABLE II.—*Part 2: Effect of Varying Salt Concentration on the Agglutination of Sensitized Bacteria.*

Concentrations of sodium chloride.

8 M/7.	4 M/7.	2 M/7.	M/7.	M/14.	M/28.	M/56.	M/112.	M/224.	M/448.	M/896.	M/1792.	Distilled water.
———	———	———	———	———	———	———	+v.s.	—	—	—	—	—
———	———	———	———	———	———	———	+s.	—	—	—	—	—
———	———	———	———	———	———	———	+	—	—	—	—	—
———	———	———	———	———	———	———	———	+s.	—	—	—	—
———	———	———	———	———	———	———	———	+	+v.s.	—	—	—
- - - -	- - - -	———	———	———	———	———	———	———	+s.	—	—	—
- - - -	- - - -	———	———	———	———	———	———	———	+	—	—	—
—	—	tr.f.	tr.f.	tr.f.	tr.	+v.s.	+	———	+	tr.f.	—	—
—	—	—	—	—	tr.f.	tr.f.	tr.	+v.s.	+v.s.	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—

s. = slight; v.s. = very slight; tr. = trace; tr.f. = faint trace.

A heavy rule = complete agglutination. A broken rule = complete agglutination with very fine flocculi remaining in suspension. A dotted underline = position of optimal agglutination.

approximate measurement, but, if very concentrated mixtures are used, antibody may be found in the supernatant even from the end-point or "C" ratio.

The examination of supernatants from a "B" series showed that with a constant serum concentration and an increase in the number of organisms from 1 × X to 8 × X (which was the "B" optimal ratio), the amount of antibody combined increased from 40 to 44 doses, so that the fall in the degree of sensitization of the individual organisms with the increase in their number was less than the fall in the serum/suspension ratio.

It is clear that antibody may unite with the bacterium in a great many different proportions, and that optimal agglutination is not related to the maximum amount of combined antibody. Marrack and Smith (1931), who also quote Heidelberger and Kendall (1929), have shown that in precipitation with soluble antigens the proportions of antibody and antigen in the precipitate may show a wide range of variation if both possess several combining groups.

The presence of free agglutinin residue, not attributed to antibody-antigen dissociation, in the supernatants from all mixtures giving complete agglutination conflicts with the conception of a state of antibody-antigen equivalence, corresponding to one or other of the optimal agglutination ratios, in which the quantities of the reagents are exactly balanced, leaving no free residue

of either after combination. Alternatively, it may be suggested that a certain antibody tension is necessary for a given degree of specific antibody adsorption, and that this tension is increased relatively for the higher degrees of adsorption, giving rise to an increasing percentage of free antibody residue. The amount adsorbed would therefore always be less than the total amount of antibody present.

In the interpretation of the supernatant results, it may be contended that the free antibody residues represent not only the excess fraction, but also some antibody dissociated from its union with the bacterium. The fact that bacteria sensitized at any but the highest serum ratios may be washed in M/7 saline without yielding any appreciable amount of free antibody is against the probability of dissociation; but it is also true that if the centrifuging of a low serum/suspension ratio mixture be deferred for 24 hours, the amount of free antibody in the supernatant will usually be less than it would have been had the centrifuging been done when the mixture was only half an hour old. This disappearance of free antibody may be due to a slow antibody-bacteria union at high serum dilutions, or, taking the other view, to a firmer antibody-bacteria union, resulting from storage, diminishing the tendency to dissociation.

It is also possible that the Flexner "Z" serum may contain multiple antibodies, some of which, corresponding to minor antigens, may be present in relative excess and remain in the supernatant. On the whole, however, a large number of supernatant tests tends to promote confidence in their value as a guide to the amount of antibody combined, when sufficient time is allowed for firm antibody union and high temperatures are avoided before centrifuging.

II. *Salt Agglutination of the Sensitized Organisms.*

The deposits from centrifuging the inital mixtures shown in the centre column of Table II were washed in M/7 saline, without complete dispersion, to remove traces of serum, and, after re-centrifuging, were then dispersed in distilled water at pH 6·8 to give suspensions containing 2×10^8 organisms in 1·0 c.c. These dispersions were tested against a series of falling concentrations of sodium chloride, commencing at 8 M/7, for salt agglutination. Table II, Part 2, shows that for every different degree of antibody sensitization, as interpreted from the results shown in Part 1, there is an *optimal salt concentration* giving the most rapid agglutination, and a minimum salt concentration below which complete agglutination does not occur. It will be noticed that the minimum salt concentration falls with the fall in the antibody sensitization of the organisms. The positions of most rapid agglutination in this table are not so sharply defined as in some other experiments, because it was not possible, on this occasion, to take the readings at the critical times. The optimal salt concentration for the "A" ratio sensitization is near to M/7.

At the end-point (serum dilution 1/640) sensitization the redispersed organisms were *completely* agglutinated only by a salt concentration of M/224, and only a trace of agglutination was given by M/7 salt, although complete agglutination occurred in the initial serum/suspension mixture at this salt concentration. This seems to be due to the loss, by separation, of a very small amount of the attached antibody during dispersion in distilled water.

As a similar effect was not caused by washing in M/7 saline, it may be assumed that the removal of other components of the serum does not play a part. The antibody is, apparently, not impaired functionally by solution in distilled water. Table IV shows that the lowest serum/suspension ratio capable of giving complete agglutination was not agglutinated completely by M/7 salt, but only by concentrations of M/112 to M/224 salt.

It may be assumed, in explanation of the optimal salt agglutinations and the minimum salt concentrations giving agglutination, in the case of the Flexner bacillus, that the amount of electrolyte necessary to reduce the surface potential below the critical level, and cause agglutination, varies directly with the amount of antibody attached.

Northrop and De Kruif (1922), studying the effect of the salt concentration on agglutination of *Bact. typhosum*, found that at the highest dilution of immune serum, 1/19,200, complete agglutination occurred only at a salt concentration of 0·10 N., and not at 0·03 N. or 0·30 N., but with rising concentrations of serum, from this point, the range of salt concentrations giving complete agglutination spread out on either side of 0·10 N. salt, and at a serum dilution of 1/1200 complete agglutination occurred between 0·01 N. and 1·4 N. salt. At still higher serum concentrations, up to 1/150, no stronger salt than 0·01 N. was necessary for complete agglutination. These results, with *Bact. typhosum*, which show a progressive increase, from 0·01 N. to 0·10 N., in the minimum salt concentration giving complete agglutination, between the point of complete antibody sensitization and the end-point, are in sharp contrast with those from the Flexner bacillus, given in Table II, in which a *fall* in the minimum salt concentration from M/112 to M/224 (in Table IV from M/56 to M/224) is shown. This difference may be explained by Shibley's (1926) observation that the surface potential of unsensitized dysentery bacilli is relatively low (3 to 5 millivolts) when compared with that of *Bact. typhosum* or *Bact. coli* (about 23 to 40 millivolts), so that in bringing the surface potential to that common to bacteria completely covered with denaturated antibody (10 to 15 millivolts), immune sensitization raises the potential in the case of the dysentery bacilli and lowers it in the case of *Bact. typhosum*. Therefore, from the point of complete sensitization to the end-point, the surface potential of *Bact. typhosum* should rise and require a progressive increase of salt for its reduction, while that of Flexner's bacillus would fall and thus require less salt.

Northrop and De Kruif also found that the " lower limiting salt concentration " of 0·01 N. was the same for all grades of antibody combination in excess of complete sensitization, but Flexner's bacillus, as shown in Table II, requires an increase in the minimum salt concentration with the progressive additions of antibody. It may be significant that Northrop and De Kruif were using an " H " antibody-antigen system, and, in the higher serum concentrations, possibly an " O " system as well.

THE INFLUENCE OF THE SALT CONCENTRATION ON THE OPTIMAL AGGLUTINATION RATIOS.

Bier (1931) showed, in the case of agglutination of a Flexner dysentery bacillus in the " A " series, that strong concentrations of salt displaced the

position of optimal agglutination to the left, to a higher serum/suspension ratio, and delayed the time of agglutination. He explains this result by the view, expressed by Northrop and De Kruif (1922), that a strong concentration of salt reduces the cohesive force between the sensitized bacteria and retards agglutination, and furthermore, that the velocity of agglutination in a particular tube is a function of the relation between the antibody/antigen ratio and the salt concentration. In the present work Bier's experiments have been repeated and his results have been confirmed, and, in addition, tests have been made of the effects of very strong and very weak concentrations of salt on all three serum/suspension ratios.

Effects on the "A" Ratio.

Using the ratios determined at M/7 salt as the standards for comparison, a concentration of 5 M/4 salt displaced the position of "A" optimal agglutination to the left to twice the former serum/suspension ratio, while M/56 salt displaced it to the right to one-fourth of the former serum/suspension ratio. In both cases agglutination was slower than at M/7 salt.

Table IV shows that progressive reduction of the salt concentration will eventually bring the position of the "A" ratio into approximation with the agglutination end-point.

The influence of the salt concentration on the quantity or the quality of antibody-bacteria union must also be considered in connection with the displacement of the position of optimal agglutination. Table III shows the effect of the salt concentration on the proportion of antibody combining in an "A" series of serum/suspension mixtures. Three tests were put up, at 5 M/4 (hypertonic), at M/7 (isotonic) and at M/140 (hypotonic) salt concentrations respectively. The supernatants were collected after standing for 24 hours in the cold and were titrated against a suspension containing 2×10^8 organisms in 1·0 c.c. The salt concentrations of the supernatants were adjusted to M/7, as far as possible, in the titrations.

TABLE III.—*Effect of Salt Concentration on the Amount of Antibody Bound by Bacteria.*

Antibody in each serum dilution before absorption.	Antibody residues in supernatants.		
	Hypertonic salt.	Isotonic salt.	Hypotonic salt.
256	24·00	60·0	70·00
128	7·00	12·0	20·00
64 "A"	5·00	3·0	8·00
32	3·00	1·5	3·00
16	2·00	—	0·75
8 "B"	1·75	—	—

The figures, in all cases, represent end-point agglutination doses of antibody for the 2×10^8 suspension.

In the left-hand column the positions of the "A" and "B" ratios are marked. These ratios were determined for the stronger suspension of 8×10^8 organisms to 1·0 c.c. used in the absorptions, and the larger agglutinin end-point doses have been converted into agglutinin doses for a suspension of

2×10^8 organisms in 1·0 c.c. for comparison with the results of the supernatant titrations; for this reason the usual 16 end-point agglutinin doses of the "A" ratio are expressed as 64 doses for the lighter suspension.

The results of this experiment, which have been confirmed on other occasions, show that, judged by the free antibody residues, the hypertonic salt, as compared with isotonic salt, gives greater antibody combination from serum/suspension ratios in excess of the "A" ratio, and lower combination at the "A" ratio and lesser serum/suspension ratios. The hypotonic salt gives lesser combination at all serum/suspension ratios. So far as they could be applied, salt agglutination tests confirmed these findings.

Regarding the quality of antibody union, tests of washing with distilled water point to a firmer binding of antibody by the strong salt at the higher serum proportions and to an increase in the cohesiveness of the flocculi.

The view that the change in the position of optimal agglutination is a function of the relation between the antibody/antigen ratio and the salt concentration is supported by the salt agglutination results shown in Table II.

In the displacement of the position of optimal agglutination by strong salt, however, the anti-cohesive action must play an important part. The earliest effect observed on raising the salt concentration above M/7 is a delay in agglutination at the "A" ratio, and further increase of the salt concentration leads to its displacement to a higher serum/suspension ratio. As judged by the velocity of agglutination, this displacement seems to be due more to depression of agglutination at the old optimal ratio by a relative excess of salt than to the development of better agglutinating conditions at the new optimal ratio. The slightly greater antibody combination given by the strong salt at the new optimal ratio and the lessened combination at the old optimal ratio, shown in Table III, are minor causes of the movement.

In the displacement of optimal agglutination by weak salt, the reduced ratio of antibody to antigen in the compound formed (Table III) would tend to cause a movement to the left, but this influence is relatively feeble, and displacement to the right is brought about by the optimal relation of a lower antibody/antigen ratio for the reduced salt concentration (Table II, part 2), and the fall in the surface potential of sensitized dysentery bacilli, from the point of complete sensitization to the end-point, suggested by Shibley's work, previously referred to, which would lead to a movement of optimal agglutination to lower serum concentrations with the progressive dilution of the salt.

The velocity of optimal agglutination at M/56 salt is less than at M/7 salt, because the antibody/antigen compound giving optimal agglutination with M/56 salt has a lower antibody/antigen ratio with, presumably, a consequent reduction in cohesive power.

Of the three salt concentrations used, 5 M/4, M/7 and M/56, the greatest velocity of agglutination occurred with M/7. In Table II, part 2, this was shown to be about the optimal salt concentration for the "A" ratio sensitization, and it has been found particularly suitable for this agglutination work because it is approximately isotonic with serum, and thus permits the use of strong concentrations of serum in a series without causing variation in tonicity, and it is, of course, necessary in the complement-fixation experiments.

The Effects on the " B " Ratio.

In a " B " series increase of the salt concentration above M/7 delays agglutination ; for example, thrice the time was required for optimal agglutination when the salt was increased from M/7 to 3 M, but there was no *observed* displacement of the position of optimal agglutination in a series of two-fold dilutions of the suspension.

Reducing the salt concentration below M/7 seemed to have no effect on the time of optimal agglutination, but it caused displacement to the left to a lower serum/suspension ratio ; a salt concentration of M/210 gave optimal agglutination at one-fourth the " B " serum/suspension ratio determined at M/7 salt, and the serum proportion of the new optimal ratio was half that of the lowest serum/suspension ratio giving complete agglutination with M/7 salt. The later stages of agglutination by M/210 salt were delayed and the reaction was checked in the direction of rising serum/suspension ratios, so that complete agglutination failed before the point corresponding to the " A " ratio in an M/7 salt series was reached.

The Effects on the " C " or End-point Ratio.

Strong salt concentrations depress agglutination at the end-point titre because of their anti-cohesive effect on the relatively feebly sensitized organisms, and also, possibly, because of the lessened antibody combination from high serum dilutions in the presence of strong salt (Table III).

Low concentrations of salt, such as M/112, give a higher end-point than M/7 salt, as is shown in Table IV. A salt concentration of M/7 seems to be relatively excessive for the smallest amount of combined antibody, or minimum sensitizing dose, capable of giving complete agglutination of the standard suspension of 2×10^8 organisms in 1·0 c.c. (Table II, part 2). This end-point antibody/antigen compound is completely agglutinated only by salt approaching the lower limiting concentration of M/224.

TABLE IV.—*Influence of the Salt Concentration on the End-point of Complete Agglutination or " C " Ratio.*

Salt concentrations.	Dilutions of serum.							
	1/75.	1/150.	1/300.	1/600.	1/1200.	1/2400.	1/4800.	1/9600.
M/7	—	—	—	—	—	—	+s.	—
M/14	—	—	—	—	—	—	+s.	tr.
M/28	—	—	—	—	—	—	+	tr.
M/56	—	—	—	—	—	—	+	+tr.
M/112	+s.	+	—	—	—	—	—	+v.s.
M/224	—	—	—	+	—	—	—	tr.
M/448	—	—	—	—	tr.	+	+	tr.
M/896	—	—	—	—	—	—	tr.	—

A light bacterial suspension was used.

An unbroken rule indicates the range of *complete* agglutination, and a dotted line the position of primary agglutination, corresponding to the displaced "A" optimal ratio.
tr. = trace ; s. = slight ; v.s. = very slight.

The factors which influence the displacement of the " A " ratio to the left and to the right by strong and weak salt concentrations respectively may also in some measure, influence the similar movements of the " C " ratio. The following end-points of complete agglutination were given at the different salt concentrations shown : a serum end-titre of 1/1500 at 3 M salt, 1/2500 at M/7 salt and 1/4500 at M/140 salt.

RESISTANCE OF THE COMBINED ANTIBODY TO WASHING WITH SALT SOLUTION AND DISTILLED WATER.

The theory of agglutination most frequently advanced is that immune body, which belongs to the pseudoglobulin fraction, unites firmly and specifically with the bacterium, and, in this union, undergoes a change from the hydrophilic or soluble state to the hydrophobic or insoluble state ; this change is usually referred to as *denaturation*. Eagle (1930) suggests that the specific character of immune body depends upon a change affecting some of the hydrophilic groups of the molecule, so that in firm attachment these groups would be directed towards the bacterium, causing the *hydrophobic* groups to face the water. In this way the surface properties of the immune body-bacterium compound are determined by the hydrophobic groups. If there is sufficient immune body present it will form a " complete film " of denaturated or hydrophobic globulin on the organism, which now becomes merely the vehicle of the immune body, and the compound will behave as a particle of denaturated globulin, relatively instable in suspension. Its surface potential, on which instability of the suspension depends, is readily discharged by weak electrolytes such as low concentrations of sodium chloride, and aggregation of the particles takes place when the surface potential is reduced below about 12 to 15 millivolts.

Eagle (1932) points out that the velocity of agglutination increases with the concentration of antibody, and reaches its maximum, apparently, when a complete surface film of antibody is formed on the organisms ; after this the further addition of immune serum is relatively ineffective.

On this hypothesis the surface film of antibody would be just complete at the " A " ratio, but in the present work it has been shown that increasing proportions of serum in excess of the " A " ratio have a retarding effect on agglutination, and, when a sufficient excess is reached, will cause an inhibitory prozone.

Accepting the *complete surface film* theory, it might be assumed that antibody combined in excess of the " A " ratio would be less firmly bound than that forming the primary film. De Kruif and Northrop (1922), referring to some experiments on washing sensitized typhoid bacilli with distilled water to remove the antibody, state that about twelve agglutinin doses were firmly bound, and antibody in excess of this amount was easily removed by washing. It is noteworthy that the organism used was a flagellated species. Huntoon (1921), on the other hand, believes that there is less justification for the assumption that antibody is attached to antigen in two distinct portions in sharply different degrees of firmness, than to assume a graduated variability in the firmness of union, the less firmly bound antibody being more easily dissociated.

Effect of Washing with Salt Solution.

Centrifuged deposits of sensitized Flexner "Z" dysentery bacilli, when dispersed in M/7 saline at room temperature, yielded *free* antibody in appreciable amount only when the organisms had been sensitized at serum proportions very much in excess of the "A" ratio. The amounts of dissociated antibody were very small, and were found only in the first washing. Huntoon (1921), de Kruif and Northrop (1922) and Eagle (1930) have all observed that combined antibody is removed only with difficulty by washing with isotonic salt solution.

In order to find the highest salt concentration at which antibody is dissociated in appreciable quantity from a suspension sensitized at the "A" ratio, a serum/suspension mixture of this ratio was prepared in M/7 saline and then divided into a number of uniform volumes, which were centrifuged after standing for one hour on the bench. Every sensitized deposit was dispersed in a uniform volume, but a different strength of salt solution—one of a series of descending salt concentrations commencing at M/7. After standing for 30 minutes these dispersions were centrifuged, and the supernatants were examined for *free* agglutinin by titration against a suspension of 2×10^8 organisms to 1·0 c.c. No free agglutinin was found when the sensitized organisms had been dispersed in salt concentrations above M/112. At this concentration a trace of free agglutinin was detected and the quantity increased to two agglutinin doses, estimated, in 1·0 c.c. of supernatant from the M/448 salt concentration, and eight agglutinin doses from the M/1798 concentration; and this amount was not exceeded by dispersion in distilled water.

Effect of Washing with Distilled Water.

For distilled water washing the following technique was followed: Serum/suspension mixtures were kept for not less than 1 hour, with occasional shaking, at room temperature to allow antibody-antigen union. They were then centrifuged, and the supernatants, if required, were kept, while the deposits were gently, but not completely, dispersed in M/7 saline up to the original volume, and then re-centrifuged to remove any free serum present. The saline washings were put aside for examination, and the washed deposits were dispersed in distilled water, at pH 6·8, up to the original volume. These distilled water dispersions, after standing for 30 minutes, were centrifuged, and the resulting supernatants constituted the *first distilled water washings*. The washing process was usually repeated, but on no occasion did the number of successive distilled-water washings exceed four. The washings were titrated against a suspension of standard density for measurement of free agglutinin.

Table V shows the results of distilled water washing following the procedure just outlined. Part 1 deals with suspensions sensitized at the "A" and "B" ratios. The "A" ratio in this case was serum 1/5 (approximately 512 agglutinin doses for an equal volume of a 2×10^8 suspension) to an equal volume of a 1/3 dilution of a 2×10^{10} suspension. Part 2 is from a separate experiment, using a different suspension, and the sensitizing mixtures were in much weaker concentration than those in Part 1.

Further washing yielded no appreciable amount of free antibody from either of the sensitized suspensions in Part 1, so that, on the assumption that

TABLE V.—*Elution of Agglutinin from Sensitized Bacteria by Distilled Water.*

	1st washing.	2nd washing.	3rd washing.
Part I :			
"A" ratio	20	30	1
"B" ratio	—	8	1
Part II :			
Four times "A" serum/suspension ratio	48	6	0·5
One half "A" serum/suspension ratio	2	4	—
"B" ratio	—	1 +	—
"C" ratio	—	tr.	—

The figures refer to agglutinin doses for a suspension containing 2×10^8 organisms in 1·0 c.c.

approximately 93 per cent. of the available antibody (about 476 agglutinin doses) had combined at the "A" ratio, the amount dissociated by washing is relatively small. Huntoon (1921) observed that only a portion of the combined antibody could be dissociated by washing, and concluded that the antibody-antigen reaction is only partly reversible.

The results given in Table V are in keeping with those of similar experiments, which show that when the organisms are sensitized at a serum/suspension ratio in excess of the "A" ratio, the greater part of the dissociable proportion of the antibody is removed with the first distilled water washing; when sensitized at the "A" ratio approximately equal amounts of antibody may be found in the first two washings, or either washing may contain a small excess of antibody over the other; when sensitized at lower serum proportions than the "A" ratio the second washing usually yields more antibody than the first, and at the "B" ratio it was exceptional, unless in the case of a very concentrated mixture, to find any appreciable amount of antibody in the first washing, but nearly all the dissociable portion of the antibody was found in the second washing. These results do not support the view that the attached antibody is held in two sharply different degrees of firmness, corresponding to the primary surface film of antibody and the excess, but merely show that some of the antibody from the excess proportion is more easily dissociated than the smaller amount forming the primary surface film. This is in harmony with Huntoon's (1921) view that there is a "graduated variability in the firmness of union", and washing will not remove all the attached antibody in excess of the "minimum sensitizing dose".

The frequent absence of an appreciable amount of antibody from the first distilled-water washing of a suspension sensitized in the "B" ratio, while the second washing removed nearly all the dissociable antibody, might be explained on the assumption that, when the volume of the washing liquid is not greater than that of the original serum/suspension mixture, salt diffusing from the sensitized organisms may reach a sufficient concentration in the washing liquid to *protect* the small amount of attached antibody from dissociating. This explanation is supported by the fact that a relative increase in the volume of distilled water used for the first washing increases the amount of antibody dissociated from a suspension sensitized in the "B" ratio, but has, apparently, no similar effect in the case of a suspension sensitized at a high serum ratio.

For example, a concentrated serum/suspension mixture in the " B " ratio was divided into four equal volumes and the deposits from centrifuging were dispersed as follows: (*a*) In 1 volume, (*b*) in 2 volumes, (*c*) in 4 volumes and (*d*) in 8 volumes of distilled water relative to the volumes of the original mixture. The total amounts of antibody liberated in these four dispersions were estimated as (*a*) 0·5 dose, (*b*) 1·5 dose, (*c*) 4 doses and (*d*) 8 doses. The centrifuged deposits from which the washings were collected were dispersed again in a similar series of volumes of distilled water, and this time the relative total of antibody liberated was estimated as 8 doses in every case. In a second experiment, in which the suspension washed had been sensitized at 4 times the " A " serum/suspension ratio, the first dispersions in the four different volumes of distilled water yielded the same amount of antibody in every case, showing that the volume of the washing liquid had no apparent influence on the amount of antibody dissociated from this highly sensitized suspension. It seems, therefore, that an increase in the volume of the washing liquid causes greater antibody-antigen dissociation only when it has the effect of reducing the concentration of salt present in the washings to less than the critical point in relation to the amount of combined antibody. Huntoon (1921) expresses the opinion that " at present the most that can be said regarding antibody-antigen dissociation is that *apparently* it is governed by the dilution of the *binding-salt* present ".

As stated previously, a small amount of antibody may be dissociated from a highly sensitized suspension in the presence of M/7 salt, but this concentration of salt will not permit any appreciable dissociation from a suspension sensitized in the " A " ratio. In the experiment described earlier in this paper, dissociation was shown to occur from organisms sensitized at this ratio when the salt concentration had been reduced to M/112. In the case of organisms sensitized at the " B " ratio, apparently, a very small amount of salt may prevent dissociation.

This work does not show at what stage antibody-antigen dissociation occurs; presumably the antibody is liberated directly from its union with the bacterium, but it is possible that some antibody present in the washing liquid may be attached to separated haptene or to fragments of bacterial substance, from which it only dissociates on the addition of the fresh suspension.

THE POSITION OF MAXIMUM COHESION OF THE FLOCCULI IN RELATION TO THE " A " AND " B " RATIOS.

If an agglutination series arranged to determine the " A " optimal ratio be allowed to proceed to complete agglutination, and then an attempt is made to disperse the flocculi by withdrawing into and discharging from a teat pipette, using, as well as can be judged, the same degree of dispersive force in every tube, the flocculi in one or more of the tubes will be found more resistant to dispersion than those in the other tubes of the series; and, occasionally, even prolonged efforts may fail to obtain a complete dispersion of the organisms. The technique, admittedly, is crude, but the results of a large number of tests have been reasonably consistent. If the tubes are returned to the water-bath after dispersion has been carried as far as possible, re-agglutination will

commence at the point which had shown the greatest resistance to dispersion, and a very sharp reading of the position of maximum cohesion may be obtained. In many of the tests maximum cohesion was found to coincide with the position of the " A " ratio, but in others it was displaced one tube to the left in a two-fold serum dilution series—that is, to twice the " A " serum/suspension ratio.

In the " A " series, complete dispersion was always easily obtained in the tube corresponding to the " B " ratio while the flocculi at the " A " ratio were still intact.

Applying the dispersion test to a " B " series, the most resistant zone was found to occur in three or four tubes embracing the position corresponding to the " A " ratio, but not including the " B " ratio.

THE INHIBITORY EFFECT OF IMMUNE SERUM IN EXCESS OF THE "A" RATIO.

It has been shown that the increasing addition of immune serum in excess of the " A " optimal ratio progressively retards the velocity of agglutination, leading to inhibition of the reaction with the occurrence of a prozone if the serum is in sufficient excess, and also lessening the cohesion of the flocculi.

Accepting the hypothesis that the surface film of antibody is just complete at the " A " ratio, antibody combined in excess of this amount must be superadded to the film and does not come into immediate union with the bacterium. Does the excess antibody combine specifically or merely as non-specific adsorption to the film of denaturated or hydrophobic antibody ? Is the excess antibody less completely denaturated than that of the primary film, thus giving rise to a less hydrophobic surface ? Or, alternatively, is the arrangement of the antibody molecules in the *excess structure* such as to present an increasing proportion of hydrophilic groups to the water surface ?

The following experiments show that the inhibitory effect of an excess of immune serum is manifest whether the excess is present in the initial mixture of serum and suspension or is added after the *complete antibody film* has formed. The " A " optimal ratio having been determined as serum 1/64 to suspension 1/13, a series of similar optimal mixtures of serum and suspension was prepared, and, after a stated interval, the excess serum was added from a

TABLE VI.—*The Set-up of Experiments on the Effect of Excess Immune Serum, Added after an Interval, on the Velocity of Agglutination.*

	Tube No.								
	1.	2.	3.	4.	5.	6.	7.	8.	9.
(*a*) Immune serum, optimal proportion; vol. 0·25 c.c.	1/64	1/64	1/64	1/64	1/64	1/64	1/64	1/128	1/256
(*b*) Suspension 1/13; vol. 0·25 c.c.	c.	c.	c.	c.	c.	c.	c.	c.	c.
(*c*) Immune serum, excess dose; vol. 0·25 c.c.	1/2	1/4	1/8	1/16	1/32	1/64	s.	s.	s.
(*d*) M/7 saline diluent, vol. 0·25 c.c.	s.	s.	s.	s.	s.	s.	s.	s.	s.

c. = suspension; s. = M/7 saline.

graduated series of serum dilutions. In the control test the excess serum was added to the optimal amount before adding the suspension. Table VI shows the arrangement of the experiment.

In the control set the reagents were added in the order of (*a*), (*c*), and then (*b*) and (*d*) together. In the five other sets (*a*) and (*b*) were mixed immediately, and (*c*) and (*d*) together were added after 5, 15, 30, 45 and 60 minutes. On completion of the mixtures, every set was incubated at 45° C., and in all six tests optimal agglutination occurred in tube 7, and the inhibitory effects were similar in all cases.

In another experiment the mixture of (*a*) and (*b*) was placed on the water-bath until agglutination occurred. The flocculi were then dispersed as completely as possible and (*c*) and (*d*) were added. On re-incubating, optimal agglutination appeared in tubes 6 and 7 ; the optimal reading was less sharply defined than in the other tests, and inhibition, although well marked in the lower serum dilutions, tended to be less evident in the higher.

Dispersion tests showed the maximum cohesion of flocculi about tube 6 in every case. Dispersion was easily effected in the zone of considerable serum excess.

It is evident, therefore, that the full inhibitory effect of immune serum excess can be obtained even when the excess is added after the formation of the *complete antibody film*.

EFFECTS OF ADDING NORMAL SERUM TO THE IMMUNE SYSTEM.

In a test arranged according to the scheme in Table VI, rabbit normal serum was substituted for the immune serum excess in row (*c*). Optimal agglutination occurred in tube 7, but the extension of the reaction to the left was more rapid than in the presence of immune serum excess. Dispersion tests showed a scarcely perceptible fall in cohesion from tube 7 to tube 4, but in tubes 1 and 2 the organisms dispersed easily. On re-incubation, primary agglutination reappeared in tube 7, but the gradations of agglutination in the other tubes were now more sharply defined.

In another experiment a uniform dose of normal serum was added to every tube in an " A " series of immune serum dilutions before adding the suspension. As compared with a control test, agglutination at the " A " optimal point was very slightly retarded without displacing the position of optimal agglutination. In the region of immune serum excess the normal serum caused a *relative* increase in the velocity of agglutination, which was most apparent where the inhibitory effect of the immune serum excess should be manifest. To the right of the " A " ratio the retarding action of normal serum became less evident, and it was not apparent at the " B " ratio. At the end-point or "C" ratio the normal serum augmented the action of the immune antibody, and carried complete agglutination a little beyond the immune serum end-point.

In order to get more exact readings of the effects of normal serum on agglutination of the immune compound, the following scheme was arranged : A series of two-fold dilutions of the immune serum, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512 and 1/1024, was prepared, and from every dilution 8 0·25 c.c. volumes were distributed into a set of 8 agglutination tubes. To

every set of 8 tubes was added a graduated series of 7 dilutions of normal serum in 0·25 c.c. volumes, commencing with a 1/2 dilution; and to the eighth tube 0·25 c.c. of M/7 saline was added. Finally every tube received a uniform dose of 0·5 c.c. of a 1/48 dilution of suspension giving an "A" optimal ratio of serum 1/128 to suspension 1/48.

Table VII illustrates the arrangement of the test in the case of the 1/4 immune serum dilution. Eight similar tests were put up, and "A" optimal proportions were given by that with the 1/64 dilution of immune serum.

TABLE VII.—*Example of the Set-up of Experiments on the Effects of Varying Amounts of Normal Serum on the Rate of Agglutination.*

	Tube No.							
	1.	2.	3.	4.	5.	6.	7.	8.
Immune serum dilutions, 0·25 c.c.	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4
Normal serum dilutions, 0·25 c.c.	1/2	1/4	1/8	1/16	1/32	1/64	1/128	s.
Suspension 1/48, 0·50 c.c.	c.	c.	c.	c.	c.	c.	c.	c.

All tests were incubated at 45° C., and the position of primary agglutination and the progress of the reaction were recorded. After 24 hours on the water-bath the deposits were dispersed and the position of maximum cohesion noted. Finally the tests were re-incubated and the order of agglutination again recorded.

In the set containing the "A" optimal proportion of immune serum, 1/64, agglutination commenced in tube 8 and spread gradually to tube 1.

Immune serum proportions higher than the "A" ratio.—In the 1/32 immune serum set, optimal agglutination was ill-defined, but the reaction was slightly stronger in the four tubes to the right than in those on the left.

In the 1/16 set there was no definite point of primary agglutination, and, apart from minor irregularities, all the reactions appeared alike.

In the 1/8 set agglutination commenced in tube 1 and spread gradually to tube 8.

In the 1/4 set the order of agglutination was the same as in the 1/8 set, but the reaction was weaker.

Immune serum proportions lower than the "A" ratio.—In the 1/128 set primary agglutination appeared in tube 8 and spread rapidly to tube 1.

In the 1/256 set agglutination was stronger in the right-hand tubes, but no optimal point could be determined.

In the 1/512 set, which gives the "B" ratio, agglutination appeared synchronously in all tubes.

In the 1/1024 set, the end-point ratio, agglutination started in tube 1 and spread gradually to tube 8.

In all of these tests the zone of maximum cohesion approximated to the position of earliest agglutination, and re-agglutination confirmed the results of the earlier reaction.

Confirmation of these results was furnished from similar tests, but at the

" B " ratio serum dilution agglutination has been found sometimes to commence in tube 1, and on one occasion in tube 8.

The following explanation of the phenomenon is offered: At the " A " ratio, adopting the hypothesis of a complete surface film of antibody, the most perfect hydrophobic surface is presented at the water-face and conditions for agglutination are optimal. The adsorption of protein from normal serum on to this surface will impair the optimal condition and delay agglutination, the delay, or inhibitory effect, being increased with the amount adsorbed.

The inhibitory and anti-cohesive effect of immune serum excess is much greater than that of normal serum, because, presumably, the immune antibody excess combines specifically and therefore more avidly, in greater quantity and more firmly with the surface film.

Passing from the " A " ratio to the zone of immune serum excess, optimal conditions cease as the antibody/antigen ratio rises, and the effect of the normal serum is no longer evident. When the immune antibody excess is sufficient to cause an appreciable degree of inhibition of agglutination, the addition of a relatively large dose of the less inhibitory normal serum reduces the inhibitory effect of the immune antibody, possibly, by interfering with the degree or the quality of its attachment to the immune compound. Inhibition will, therefore, be strongest in tube 8 and will diminish towards tube 1. With further increase in the immune serum excess the influence of the normal serum becomes less evident as its proportion falls with the increase in the quantity of immune antibody.

The experiment shows that normal serum, present in an optimal immune-serum/suspension mixture, tends to *inhibit agglutination* and to *reduce cohesion*, but in the presence of an excess of immune antibody it *lessens inhibition* by the immune antibody excess and thus *increases cohesion*.

In the case of immune serum proportions less than the " A " ratio, the inhibitory effect of added normal serum diminishes with the movement away from the " A " ratio. About the immune serum end-point, where, presumably, the antibody film is incomplete, normal serum, in a dilution at which it is unable of itself to cause agglutination, will augment the action of the small end-point amount of attached antibody, and it may even carry complete agglutination a little beyond the immune serum end-point. It may be assumed that adsorbed material from the normal serum fills the deficiencies in the antibody film and thus presents a better surface for agglutination.

It is interesting to observe that Sobotka and Friedlander (1928), working with specific pneumococcus polysaccharide and a purified precipitating antibody, found " that the addition of normal horse-serum increased the sensitivity when the concentration of antibody was low ", and raised the antibody dilution end-point, as in the agglutination experiments recorded above.

In the case of a " B " series of agglutination mixtures, the addition of a uniform dose of normal serum had no observed effect on the " B " optimal ratio, but it improved agglutination slightly at a lower serum/suspension ratio corresponding to the end-point in an " A " series, and very markedly at high serum/suspension ratios, above the " A " ratio, corresponding to serum excess in an " A " series.

RELATION OF THE "A" RATIO TO THE DEGREE OF COMPLEMENT-FIXATION.

Tests were made to find if the complement-binding power of antibody-antigen compounds formed at the " A " ratio was as great as that of compounds formed at higher serum/suspension ratios.

Briefly, the technique used was as follows: The M.H.D. of complement was determined, by the usual procedure, in the presence of a dose of bacteria equal to that used in the tests. The sensitized antigen was prepared by titrating a constant dose of bacterial suspension against falling concentrations of immune serum. Large quantities were used, and the mixtures were kept on the bench, with occasional shaking, for 1 hour and then centrifuged. The deposits were collected, washed lightly in M/7 saline to remove traces of serum, re-centrifuged and finally dispersed to a stated density in M/7 saline.

In one series of tests (Table VIII*a*) successive dilutions of the different sensitized suspensions were titrated against a fixed dose of complement. In the other series (Table VIII*b*) the amount of sensitized suspension was kept constant and the dose of complement varied. Incubation at 39° C. for

TABLE VIII.—*Complement-fixation at Varying Grades of Antibody Sensitization.*

(A)

Serum/suspension ratios in sensitization. Serum.	Suspension.	Dilutions of the sensitized suspensions. 1/12.	1/16.	1 21.	1 29.	1/36.	1/50.
1/2	1/12	———	———	———	———	———	———
1/6	1/12	———	———	———	———	———	———
1/18	1/12 (A)	———	———	———	———	———	———
1/54	1/12	———	———	———	———	———	———
1/108	1/12	———	———	———	———	———	———
1/216	1/12	———	———	———	———	———	———

(B)

Serum/suspension ratios in sensitization. Serum.	Suspension.	M.H.D.'s of complement to a 1/24 dilution of the sensitized suspensions. 8.	5·24.	3·5.	2·5.	1·9.	1·5.
1/2	1/12	..	..	..	———	———	———
1/6	1/12	..	..	..	———	———	———
1/18	1/12 (A)	..	..	..	..	———	———
1/54	1/12	..	..	..	..	———	———
1/108	1/12	..	..	..	..	..	———
1/216	1/12	..	..	..	..	..	*Nil*

The figures in the left-hand column indicate the ratios of serum to a 1/12 dilution of suspension used in sensitizing. The position of the "A" optimal ratio is marked.

A thick rule indicates the range of *complete* complement-fixation, and a lighter rule the diminishing degrees of incomplete fixation.

In part (A) various dilutions of 1/12 sensitized suspensions are titrated against a constant dose of complement.

In part (B) various doses of complement are titrated against a 1/24 dilution of the sensitized suspensions.

45 minutes was allowed for complement fixing before adding the sensitized red blood-cell suspension. Readings were taken 1 hour later and again after 18 hours in the ice-chest.

The results of several complement-fixation tests, using different samples of Flexner "Z" serum, have shown that from the end-point sensitization to the neighbourhood of the "A" ratio there is a progressive and fairly uniform increase in complement-fixation. About the "A" ratio, however, this increase is usually abruptly checked, and no further rise is observed until a much higher ratio of sensitization has been reached. Beyond this, in the limited range of higher sensitization obtainable, there is very little, if any, further increase.

In preparing the sensitized antigen suspensions, it was found usually impossible, after centrifuging, to get a complete dispersion in the neighbourhood of the "A" ratio, in M/7 saline. This resistance to dispersion, corresponding to the zone of maximum cohesion, diminished on either side of the "A" ratio, so that apparently complete dispersion could be obtained easily at relatively strong serum sensitizations. The persistence in the form of small granular flocculi of the immune compound in the neighbourhood of the "A" optimal ratio reduces the complement-fixing surface, and probably accounts for the depression of fixation in this zone. Goldsworthy (1928) has described a similar phenomenon in complement-fixation by immune serum precipitates.

It is clear, therefore, that these results do not give the full complement-fixing power of the "A" ratio immune compound, and it is not possible to state whether the "A" ratio is included in a maximum fixation zone or not.

GENERAL DISCUSSION ON THE RATIOS AND THEIR POSSIBLE APPLICATIONS.

The "A" Ratio.

It has been mentioned that the "A" optimal ratio test is analogous to Ramon's method of measuring the optimal neutralizing ratio of diphtheria antitoxin to toxin, but, as it has not been shown that the reactions between agglutinin and bacteria furnish a *complete* analogy to those between antitoxin and toxin, it has been deemed advisable to use the designation "A" optimal ratio in place of "Ramon optimal ratio". A similar remark applies to the case of the "B" optimal ratio.

The "A" ratio does not correspond to the maximum amount of attached antibody, but the evidence, especially that from the experiments with addition of normal serum and immune serum in excess and the statements of other workers quoted, suggests that it is the stage in which the antibody just forms a complete film on the organism. It is either coincident with or near to the antibody/bacteria ratio giving maximum cohesion of the agglutinated organisms, and to this, no doubt, is due the rapidity of the later stages of agglutination. Its apparent position is influenced by variations in the concentration of agglutinating salt, but, as the greatest velocity of agglutination, indicating optimal conditions, occurs with a salt concentration of about M/7, the optimal serum/suspension ratio recorded at this salt concentration should be accepted as the true "A" optimal ratio. As exemplified in the Ramon

test, its value would consist in giving a measure of the smallest amount of antibody which will completely neutralize (or cover ?) every antigenic particle. It seems to imply absorption of only about 93 per cent. of the total antibody, so that it would not be suitable as a guide to the appropriate dose of suspension for use in fractional antibody absorption.

The " B " Ratio.

When a fixed concentration of immune serum is titrated against a series of increasing concentrations of bacterial suspension, the degree of sensitization of the individual organisms falls with the increase in their number. There are, therefore, two opposing factors affecting the velocity of agglutination : (1) The increased incidence of contacts between the sensitized organisms, proportionate to the increase in their number, which accelerates agglutination ; and (2) the fall in the degree of sensitization or cohesiveness of the individual bacilli, with the fall in the serum/suspension ratio, which delays the reaction. Up to a certain point the effect of the increased contacts, first between sensitized organisms and later between the growing flocculi, exceeds that of the reduced cohesiveness, and the velocity of agglutination increases with the concentration of the suspension until the acme is reached at the " B " optimal ratio ; beyond this the advantage of the increased contacts becomes less evident and the velocity of agglutination falls. Ultimately, complete agglutination fails when the increase in bacteria has reduced the serum/suspension ratio below the end-point ratio.

The advantage in velocity of agglutination from an increase in the bacterial ratio is proportional to that increase, but the fall in the degree of sensitization of the individual organisms, as judged by examination of the supernatants (p. 27), is less than the fall in the serum/suspension ratio. It may be accepted that the maximum velocity of agglutination of the " B " ratio depends upon an optimal number of bacteria for the highest incidence of effective contacts between the sensitized organisms.

With the same reacting system the " A " and " B " ratios are in a constant relation, so it is possible to determine in a " B " series the point corresponding to the " A " ratio. It has been observed that a steep fall in velocity of agglutination occurs when this point has been passed in the direction of rising serum/suspension ratios, apparently because the inhibitory effect of antibody excess is now added to the effect of the fall in the number of organisms. Dispersion tests show that in a " B " series maximum cohesion does not coincide with optimal agglutination, but approximates to the neighbourhood of the " A " ratio point.

The " B " ratio, in the case of the Flexner " Z " system used, implied one-eighth of the proportion of serum to suspension of the " A " ratio, and, assuming that the surface film of antibody on the organism is just complete at the " A " ratio, this would mean that a little more than one-eighth of the surface is covered at the " B " ratio ; which may render this ratio unsuitable as a guide to the *neutralizing* dose of antibody. It, however, involves a higher percentage of antibody absorption than the approximate 93 per cent. of the " A " ratio, and so would be more suitable for determining the dosage of

bacteria to be used in fractional absorption, but in this respect it may owe its value to its proximity to the " C " ratio. " B " ratio determinations should be made at a salt concentration of about M/7.

The " C " or End-point Ratio.

It may be assumed that at the serum end-point of complete agglutination every organism carries the minimum sensitizing dose or the smallest amount of antibody which will cause agglutination. The true end-point, that is, complete agglutination at the highest serum dilution, appears to be given only by an optimal concentration of salt, which may vary with different species. According to Northrop and De Kruif, this is M/10 sodium chloride in the case of *Bact. typhosum*, and in the present work it was found to be about M/112 to M/224 sodium chloride for Flexner's dysentery bacillus. This difference in the optimal salt concentrations may be explained by Shibley's observation, which suggests that at the end-point antibody/bacterium ratio, the surface potential is much higher in the case of *Bact. typhosum* than in that of *Bact. dysenteriæ*. The " C " ratio, properly determined, may be a guide to the appropriate dosage of bacteria to be used in absorption reactions.

In conclusion, it is necessary to emphasize that all of the observations recorded in this paper, except those referred to in Table I, are based on work with a single strain of *Bact. dysenteriæ* Flexner " Z ", and it should not be inferred that they apply equally to other species.

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ON THE QUESTION OF PRESSOR BODIES IN THE BLOOD OF HYPERTENSIVE SUBJECTS.

O. L. V. S. DE WESSELOW AND W. J. GRIFFITHS.

From the Laboratories of the Medical Unit, St. Thomas's Hospital, London.

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THE question of the pressor bodies in the blood of hypertensive patients has been long under discussion, and it is impossible to summarize the whole literature of the subject in the present communication. Of workers in this field, some have employed the perfusion of isolated organs as a test for the presence of such bodies; some have searched for specific pressor bodies such as adrenalin or pituitrin by appropriate physiological methods; others have utilized the intact anæsthetized animal as a test object. The last method has been used by relatively few investigators.

Among these Danzer, Brody and Miles (1925) tested the effect of whole blood on the blood-pressure of the cat; they claim to have demonstrated both pressor and depressor bodies in the blood of hypertensives. The depressor effect was attributed by them to the blood proteins, since it could be eliminated by previous desensitization of the animal. It was also absent in vagotomized animals. They used a dose of 12–18 c.c. of blood, and appear to have examined only three or four hypertensive bloods.

Curtis, Moncrieff and Brody (1927), working on the same lines, found that heparinized rabbit's blood injected into cats intravenously produced a marked fall in blood-pressure with bradycardia. The fall was produced by blood or plasma, could be partially prevented by atropin or double vagotomy and eliminated by desensitization. Testing the whole blood of hypertensives on desensitized decerebrate animals with low arteriole tone, they were unable to obtain any evidence of the presence of a pressor body in five patients.

Bohn (1931) has used alcoholic protein-free extracts of citrated whole blood on cats anæsthetized with ethyl-urethane and curarized. The dose of extract in the majority of his experiments corresponded to 10 c.c. of the patient's blood. The blood extracts of normals and red hypertensives produced a fall in blood-pressure, while those of pale hypertensives were definitely pressor. A pressor effect was also obtained with the blood extracts of many acute nephritics, chronic nephritics with hypertension, and eclamptics. His investigation covered 174 cases of hypertension and 68 normals, and the above reactions were obtained in 90 per cent. of the cases.

While our work was in progress, Marx and Hefke (1933) published results obtained by a new method. They have tested out the effect of alcoholic extracts of blood on unanæsthetized dogs trained to lie still for 2 to 3 hours. The blood-pressure was taken by a Riva-Rocci apparatus at frequent intervals; the alcoholic extract was given intravenously or intra-arterially in doses corresponding to 100 c.c. blood. They obtained negative results with the

blood of normals, arteriosclerotics and essential hypertensives, while the blood of patients with secondary contracted kidneys and acute nephritis produced a high and prolonged rise in the animals' blood-pressure. There was no correlation between the height of the patient's blood-pressure and its content in pressor substance, the blood of acute nephritics with systolic pressure of only 140 mm. Hg. showing a marked effect, while that of arterio-sclerotics with pressures of over 200 mm. was absolutely negative.

Hoffmann and Anselmino (1931), who confined their studies to the hypertension of eclamptic toxæmia, have examined the ultrafiltrates of the plasma of patients suffering from this condition. The plasma was brought to a pH of 4–4·5 and ultrafiltered through collodion-impregnated candles. The filtrate was neutralized and, tested on rabbits, was found to possess both pressor and anti-diuretic qualities. The amounts of the pressor and anti-diuretic substance present varied independently of each other. In all patients with a blood-pressure above 180 mm. Hg. the pressor body could be demonstrated. In view of its sensitiveness to alkali and to ultra-violet light, its absorbability on talc, and the fact that, like pituitrin, one injection partially immunized the animal against a second, they suggested that the body was pituitrin—an interesting conclusion, in view of the known increased activity of the pituitary in pregnancy.

METHODS.

In the preparation of extracts in the search for a pressor body of unknown composition, it would seem advisable to subject the blood or plasma to as slight change of reaction as possible, and to avoid all procedures which might lead to chemical changes. For such a purpose the method used by Bohn seems to us excellently adapted, provided that the hypothetical substance is soluble in considerable strengths of alcohol, and we have therefore used it with slight modifications in the major part of our work. Briefly the freshly-drawn citrated blood was precipitated with 9 volumes of absolute alcohol, well shaken and allowed to stand for at least 5 hours on ice, and filtered. The filtrate was taken down *in vacuo* at 40° to 20 c.c., and reprecipitated with 2 vols. of alcohol. The filtrate from this precipitation was evaporated *in vacuo* at 40° to a few c.c. and made up with saline to 5 c.c. before injection. For plasma the same process was employed, except that 2 vols. only of alcohol were used for precipitation and the alcoholic extract was not reprecipitated. The extracts thus obtained showed at most a faint trace of protein and their pH was approximately 8·5.

We have also used extracts prepared by the ultrafiltration method suggested by Hoffmann and Anselmino. The plasma of heparinized blood is brought to a pH of approximately 4·5 with N acetic acid. It is then ultrafiltered by water pump through a filter candle impregnated with 7 per cent. collodion in acetic acid (Bechold, 1929), evaporated to small bulk *in vacuo* over $CaCl_2$ and neutralized with NaOH immediately before injection. Such ultrafiltrates are absolutely protein-free.

In a few cases we have deproteinized the blood by heating to 100° in acidified saline, taken down the filtrate *in vacuo* at 40° to dryness, washed the residue twice with acetone, and dissolved it in 5 c.c. of acid saline, neutralizing

before injection, as in the preparation of pituitrin from the pituitary gland. We have also precipitated the plasma with alcohol as in Bohn's method after initial acidification to 4·5 with acetic acid.

The question of dosage is obviously important. The cats used had an average weight of about 3 kg., and may be assumed to contain about 300 c.c. of blood. If the cat's vascular system is as sensitive as that of the human being to the assumed pressor body, a certain concentration of that body must be attained in the cat's blood if a significant pressure rise is to be elicited. As a routine we have used 40 c.c. of the patient's whole blood for the alcoholic

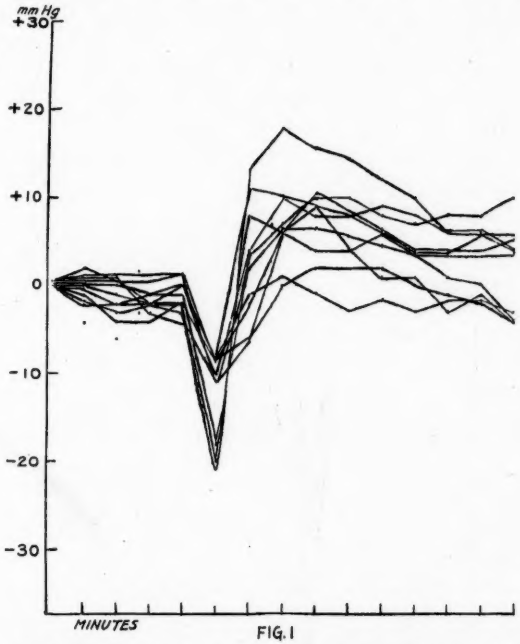

FIG. 1.—Effect on blood-pressure of the cat of alcoholic extracts of blood of normal subjects.

extraction, the filtrate obtained being equivalent to about 30 c.c. of blood. The plasma extracts have corresponded on the average to 20 c.c. plasma. Even with such doses, we can at most hope to produce a concentration of the substance in the cat's blood equivalent to 1/10 of that in the patient's blood. In a few instances extracts corresponding to 100 c.c. of blood were injected.

Since, even with the size of dose used by us, no very great rises in pressure were to be expected, every effort was made to secure a steady blood-pressure in the animal before making the injection. As an anæsthetic 2 gm. of urethane per kg. were given subcutaneously 6 hours before the experiment. A little chloroform was occasionally needed in addition. To avoid any asphyxial rise of blood-pressure due to accumulation of mucus in the air-passages, the animal

was tracheotomized, and a cannula inserted into the trachea. Artificial respiration was found to be unnecessary. Some time was allowed to elapse between the operative procedure and the start of the injections, in order to be certain that the blood-pressure was at a steady level. The pressure was recorded in the common carotid, and the injections made into the femoral vein. The various extracts and ultrafiltrates were invariably made up to 5 c.c. before injection. The sensitiveness of the animal to pressor bodies was tested by adrenalin injection. As far as possible, the same animal was used for a normal as well as for a hypertensive blood.

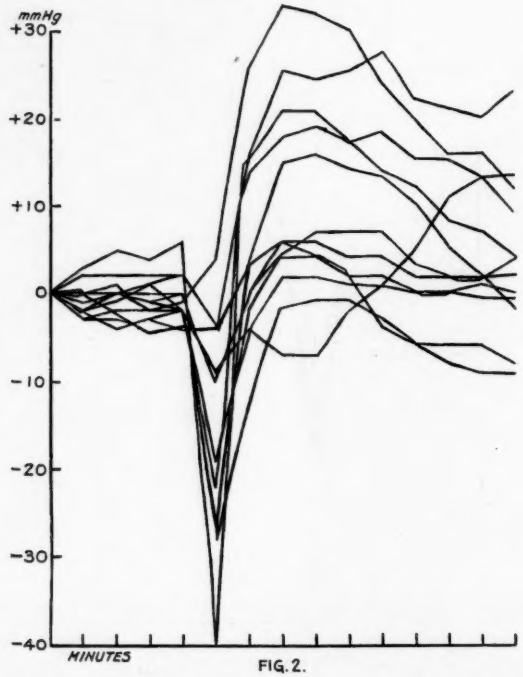

FIG. 2.—Effect on blood-pressure of cat of alcoholic blood extracts of patients suffering from essential hypertension.

MATERIAL.

Our material consisted of 30 cases of essential hypertension, 13 malignant hypertensives, 8 chronic glomerulo-nephritics, and 2 cases of pre-eclamptic toxæmia. We class as malignant hypertensives those cases which show, in addition to a high blood-pressure, albuminuric retinitis; all had albuminuria, but the maximum blood-urea of the group, when examined, was 63 mgm. per 100 c.c. They appear to correspond closely with Bohn's "pale" hypertensives. The "essential" or "red" hypertensives showed intact renal function and no

retinal change beyond early arterial degeneration. The systolic pressure of the patients examined varied from 170 mm. Hg. to 278, the diastolic from 96 to 176. Blood was taken at noon, 4 hours after the last meal.

RESULTS.

The effects of various preparations on the cats' blood-pressure were computed by means of graphs. Curves were obtained by measuring the height of the

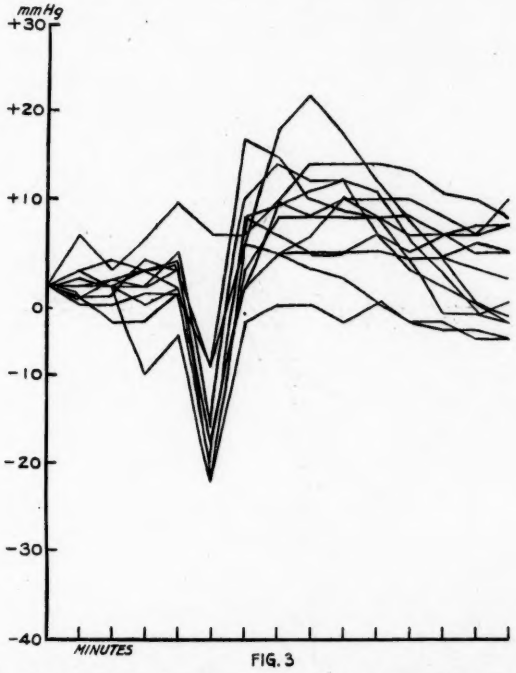

FIG. 3.—Effect on blood-pressure of cat of alcoholic blood extracts of patients suffering from malignant hypertension.

pressure tracing above the base-line at minute intervals, and plotting against the initial pressure. The absolute height of the initial pressure in the animals employed varied from 54 to 122 mm. Hg., the mean being 89. In the great majority of instances the initial pressure lay between 70 and 100.

We give figures showing the results produced by injections of alcoholic blood extracts of normals (Fig. 1), essential hypertensives (Fig. 2) and malignant hypertensives (Fig. 3) as an illustration of our findings. The injections were made at the fourth minute. In view of the negative results of our work, the publication of the curves obtained from plasma and other extracts is unnecessary.

The most striking effect of the whole blood extracts is a rapid and transitory fall in blood-pressure, lasting less than 2 minutes, and accompanied by bradycardia. This is almost invariably present with the whole blood extracts irrespective of the type of patient studied, while with the plasma extracts it is less marked, and frequently absent. The plasma ultrafiltrates also fail to produce a fall. In agreement with Bohn we find that atropin considerably diminishes the extent of the fall, while bilateral vagotomy appears to have little effect. The differences between the curves obtained with blood and plasma extracts suggest that the substance responsible for the fall is derived mainly from the red blood-corpuscles. We could find no relationship between the extent of the fall and that of the subsequent rise in blood-pressure. The depressor body appears to be present in almost all the whole blood extracts examined, both normal and hypertensive. We were unable to confirm the findings of Bohn as to the absence of a depressor factor in the blood of malignant hypertensives, and its presence in normals and essential hypertensives.

As far as the evidence for the presence of pressor bodies is concerned, the injection of 5 c.c. of Tyrode solution of itself produces a slight rise of blood-pressure in the animal, usually of from 5 to 10 mm. Hg.—an effect closely paralleled by the rise of pressure which follows the injection of alcoholic extracts of normal whole blood and plasma. As can be seen from the figures, the pressor effects of the whole blood extracts of the malignant hypertensives are not appreciably greater than those of the normal whole blood controls. The only whole blood extracts which suggest the presence of a pressor body are those of the essential hypertensives. In this group of cases, while some extracts give curves approximating to the normal, a proportion appear to produce an abnormal rise of blood-pressure in the animal. Even in these cases, however, the corresponding plasma extracts gave no definite evidence of pressor activity. The majority of the patients showing these high curves were elderly, their blood-pressures were not peculiarly high, and, as stated above, their renal function was intact. Clinically they were indistinguishable from the hypertensives, whose blood-extracts gave curves falling within the normal range.

In 4 essential hypertensives and 2 normals alcoholic extracts corresponding to 80 c.c. of whole blood were injected. The depressor effect appeared to be no greater than with the smaller dosage normally employed ; the pressor effect exceeded that obtained from the two normals in two instances, but the curves all fell within the range shown in Fig. 2. There seemed, therefore, no advantage to be gained by using larger quantities of blood than those we have employed. We have also in two cases of hypertension tested out the effect of doses smaller than our routine. Injections of alcoholic extract corresponding to 10, 20 and 40 c.c. of whole blood were made successively into the same animal. The most pronounced pressor and depressor effects in the series were obtained with the 40 c.c. dose. It was for this reason that we have used a quantity of alcoholic extract corresponding to 40 c.c. blood.

No difference could be detected between the effects produced on the animals' blood-pressure by the alcoholic extracts of plasma of normal patients and of essential hypertensives, malignant hypertensives and chronic glomerulo-nephritics.

The ultrafiltrates examined showed no pressor effect, with the exception

of one chronic glomerulo-nephritic, with slight renal failure (blood-urea 65 mgm. per 100 c.c.).

We have only had the opportunity of examining two cases of pre-eclamptic toxæmia. In both of these 20 c.c. of ultrafiltrate, concentrated *in vacuo* to 5 c.c., gave no evidence at all of the presence of a pressor body, though the blood-pressure in both patients exceeded 180 mm. Hg. at the time of examination.

The other methods of extraction employed gave entirely negative results.

DISCUSSION.

Our results appear to be in direct disagreement with those of Bohn. If we can claim to have demonstrated a pressor body in any of the types of blood or plasma extracts studied, it is to be found in the alcoholic extracts of the whole blood of essential hypertensives, a type of hypertensive blood which, according to Bohn, is destitute of pressor activity. No pressor effect was seen on injecting the blood extracts of malignant hypertensives or of chronic glomerulo-nephritics with hypertension. Further, the depressor effect of alcoholic blood extracts, which Bohn believes to be characteristic of the blood of essential hypertensives and normals, is in our experience equally marked in the other groups. Our results in no way suggest that any type of hypertension is due to the absence of a depressor substance.

We have investigated the behaviour of certain known physiological pressor bodies under the conditions of extraction employed. Pituitrin (Burroughs Wellcome's infundin) added to blood or plasma is recoverable in the alcoholic extracts as prepared by us, though it seems that recovery is not quantitatively complete. Pituitrin is readily ultrafiltered from acid saline through our collodion-impregnated candles, the recovery being quantitative. If, however, pituitrin is added to plasma at a pH of 4·5, the ultrafiltrate is completely destitute of pressor effect. The pH was adjusted immediately after the addition of the pituitrin to the plasma by adding normal acetic acid. This finding has an obvious bearing on the suggestion that pressor bodies obtained from plasma by ultrafiltration may be pituitrin.

Adrenalin ultrafiltered readily from saline, but if adrenalin was added to plasma and ultrafiltered, no evidence of pressor activity could be demonstrated in the ultrafiltrate. In view of the well-known susceptibility of adrenalin to oxidation, it is improbable that it would resist the procedure involved in the preparation of the alcoholic extracts.

As regards guanidin and its derivatives, the quantity of these bodies which could have been injected into the animals was too small to account for such pressor effects as were observed. The highest concentration of guanidin found by Major (1927) in the blood of hypertensive patients was 2·3 mg. per 100 c.c. Hence with the 40 c.c. of whole blood used by us, we should at most have injected about 0·8 mg. of guanidin, or approximately 0·2 to 0·3 mg. per kg. of the animal, whereas Major, injecting methylguanidinsulphate into dogs found it necessary to give doses of 10 mg. per kg. in order to obtain a sustained blood-pressure rise.

Even if the curves obtained from the alcoholic extracts of the whole blood

of essential hypertensives suggest that a pressor body is present, the curves from the corresponding plasma extracts do not confirm this conclusion. Alcoholic extracts of whole blood almost without exception produce a marked fall in the animals' blood-pressure, whereas the plasma extracts are little or not at all depressor in action. Further, if active pressor bodies are actually present in these bloods, it is reasonable to suppose that they are present in the plasma and not confined to the corpuscles. For these reasons whole blood extracts, as commonly employed, seem to us to carry the grave disadvantage of a depressor action with a possible resulting pressor reaction in the animal, while we cannot conceive that a pressor body confined to the corpuscles can exercise an active influence on the vascular system.

As far as our results carry us, we do not feel justified in concluding that with these methods we have obtained any evidence of pressor bodies in the blood of hypertensive patients.

One of us (W. J. G.) is indebted to the Medical Research Council for a part-time grant.

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THE PROPAGATION OF THE VIRUS OF INFECTIOUS LARYNGO-TRACHEITIS ON THE CHORIO-ALLANTOIC MEMBRANE OF THE DEVELOPING EGG.

F. M. BURNET.

From the National Institute for Medical Research, Hampstead, London.

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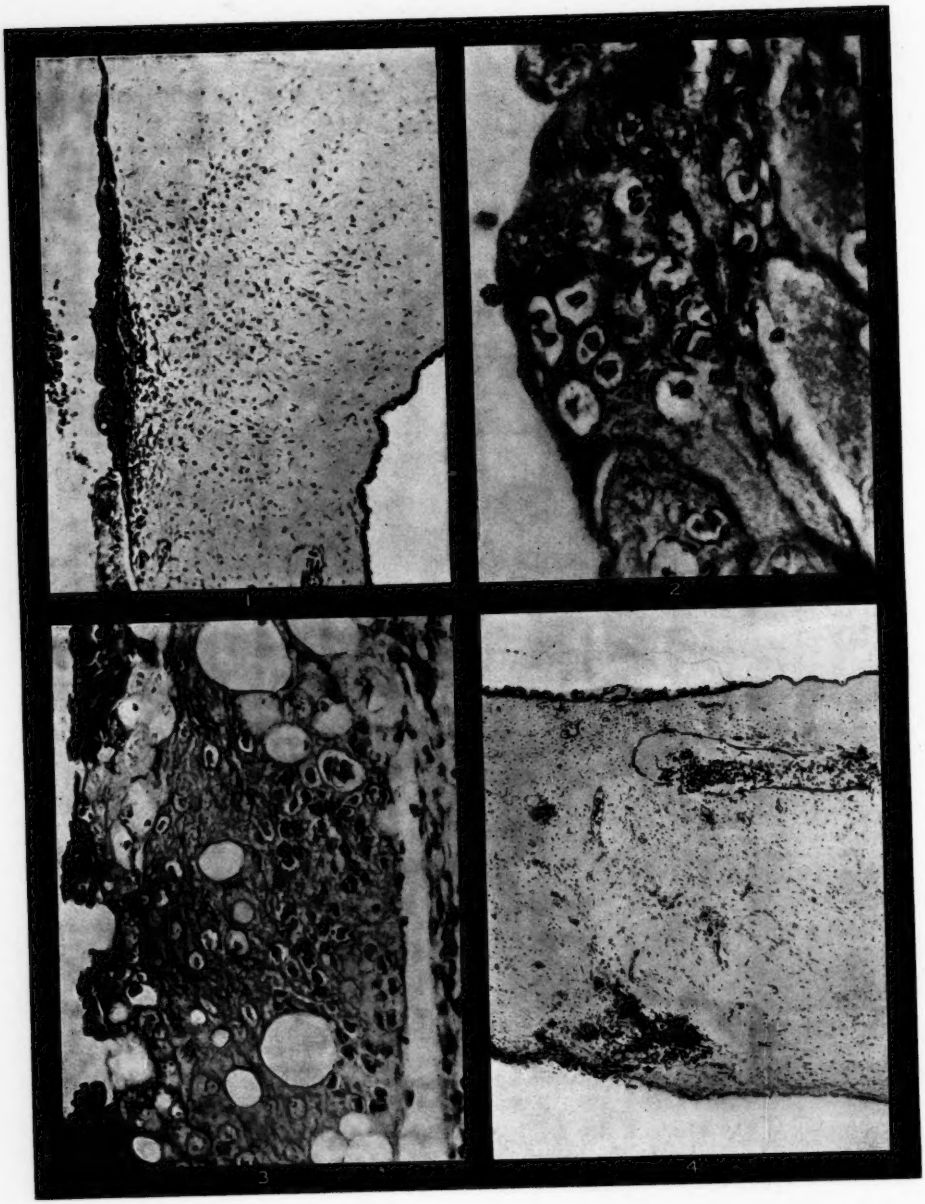
The purpose of the present note is to indicate briefly that the virus of laryngo-tracheitis can be readily propagated on the chorio-allantoic membrane of the developing egg. It is possible that the method, allowing as it does the accumulation of supplies of bacteriologically sterile virus material, may be of value to those concerned with research into or control of the natural disease.



Burnet.

UNIV. OF MICH.

The virus was obtained through the kindness of Dr. J. R. Beach, and was received as a dried powder labelled (C 10 Lot 57). A broth emulsion of this injected intratracheally into a half-grown chick induced the typical disease. The chick killed on the seventh day showed a large caseous cast almost blocking the larynx, and a separating "false membrane" lining most of the lower trachea. Three weeks later material from this chick preserved in 50 per cent. glycerol was inoculated intratracheally to chicks 18 and 19, both of which showed typical symptoms. Chick 19 was killed on the 4th day, and the swollen mucosa of the trachea scraped off, ground with quartz powder and broth, and filtered first through a sand and paper pulp filtrate and then through a permeable gradocol membrane (Elford, 1931) of A.P.S. 0·8μ.

This filtrate was inoculated by a modification of Woodruff and Goodpasture's technique, which has been previously described (Burnet, 1933), on to the chorio-allantoic membrane of four 10-day-old embryos. The eggs were opened on the 5th day after inoculation. Two were apparently recently dead

TABLE I.—*Egg Passage of Laryngo-tracheitis Virus.*

Egg.	Source of inoculum.	Macroscopic lesion.	Microscopic findings.	Intratracheal inoculations.
227	Filtrate 0·8μ tracheal lesion CH. 19	++	..	..
		++	Incl. + ecto. + endo.	..
240	Pooled 227–229	++	Incl. + ecto.	..
249	240	++	Incl. ++ ecto.	Pooled. Typical fatal lesion Ch. 24 (3rd passage).
250		++	..	
253	Pooled 249–250	++	Incl. + ecto.	..
254		++	..	..
258	Pooled 253–254	++	Incl. + ecto.	..
264	Dried lesion of 258	+	..	..
272	264	+	..	Pooled. ¾ chicks showed typical mild lesions without symptoms (7th passage).
274		++	..	

EXPLANATIONS OF FIGURES.

FIG. 1.—Early lesion, 48 hours, showing ectodermal proliferation not yet necrotic and cellular accumulation in mesodermal layer. Egg 258 (× 130).

FIG. 2.—High-power (× 1250) microphotograph of the proliferating ectodermal epithelium in fig. 1, showing early stages of intranuclear inclusions. In several cells the darkly stained nucleolus can be seen apparently being pushed to the side by the development of the less deeply stained inclusion body.

FIG. 3.—An area of unusually massive ectodermal proliferation showing a characteristic clump of nuclei, every one of which contains a well-developed inclusion. In several nuclei the piling up of chromatin on the nuclear membrane is well seen. Egg 249 (× 450).

FIG. 4.—Typical later lesion showing ectoderm on left and endoderm on the right. The ectoderm has mostly disappeared and been replaced by œdematous inflammatory tissue, but the remains of proliferated and necrosing ectoderm are visible toward the top. The endoderm shows a mild grade of proliferation, and under higher power the nuclei contain inclusions. This was the only section in which intranuclear inclusions were found in the endodermal layer. Egg 229 (× 67).

and were sterile on culture; the other two contained living embryos, but all four showed distinct lesions of the chorio-allantoic membrane. From the two living embryos emulsions of the membrane lesions were made, and used to initiate the series of egg passages shown in Table I.

THE PATHOLOGY OF THE INFECTION IN THE CHORIO-ALLANTOIC MEMBRANE.

There is a good deal of variation in the macroscopic appearances of the lesions, but the most typical form at the 4th or 5th day after inoculation is probably that of a plaque of greyish thickening in the membrane, surrounded by a zone of whiter appearance about a millimetre wide. In some cases there are several such 2-zoned plaques. The typical double edge may be lacking in some cases, and the lesion is usually complicated by œdema and varying degrees of cellular infiltration of the membrane, apart from the changes in the immediate vicinity of the typical lesions. There is never the massive proliferation which is characteristic of fowlpox and vaccinia lesions.

Histologically the lesions appear to represent primarily the response of the ectodermal epithelium on which the virus is deposited, with secondary changes in the other layers. In a section taken 48 hours after inoculation (see fig. 1) the ectodermal epithelium shows patches of proliferation with as yet little necrosis. The mesodermal region is swollen with œdema and cellular accumulation, mainly of fibroblastic type. The endodermal epithelium is somewhat hypertrophied, but shows no specific changes.

The most characteristic feature of the ectodermal lesions is the presence of intranuclear inclusions closely resembling those found in the tracheal lesions of the natural disease (Siefried), 1931. Like these they are rather patchily distributed through the sections and are not invariably present, although, judging from experience with the present rather irregularly virulent strain, they are much more readily found in the egg lesions than in tracheal ones. In both situations it is characteristic to find small foci of proliferating cells, all the nuclei of which contain typical inclusions.

The early nuclear changes are well shown in fig. 2. The large, deeply staining nucleolus seems to be characteristic of infected nuclei in the earliest stage. The inclusion develops alongside this, and as it enlarges, appears to push the nucleolus over to the nuclear membrane. The fully developed inclusions are seen in fig. 3, where the proliferating epithelium is commencing to undergo vacuolation and necrosis. The inclusion occupies the central region of the nucleus, and appears to cover about $\frac{1}{3}$ to $\frac{1}{2}$ its area, an empty space lying between it and the nuclear membrane, on which are piled up the masses representing the nucleolus and other chromatin of the initial stages. In a later stage still, where the cells are apparently dead and being removed by inflammatory cells, the nuclei are shrunken, and the acidophilic mass appears to fill the whole nucleus.

As the lesion develops, the affected ectodermal epithelium undergoes vacuolation and necrosis. The tissue is then absorbed, and its place taken by fibroblastic inflammatory tissue. This results in the characteristic double-edged plaque described above and the histological appearance shown in fig. 4. In this section one can observe the more or less normal ectoderm above running

into the necrotic remains of a region of proliferation, within which an occasional badly-preserved intranuclear inclusion may be found. Beyond this all trace of ectoderm has disappeared, its place being taken by œdematous inflammatory tissue ; as a rule the endoderm shows nothing beyond a little proliferation, but the section figured was unique in that nearly all the endodermal cells in the region shown contained intranuclear inclusions. Since the tracheal epithelium is a structure of endodermal origin, and the only other receptive zone in the fowl appears to be the cloaca, also endodermal in origin, one would have expected to find endodermal lesions more frequently in the egg. Probably the predominance of ectodermal changes is to be related mainly to the method of inoculation.

INFECTIVITY OF EGG LESIONS.

Material from the 3rd and 7th egg generation of virus was inoculated intratracheally into chickens. An emulsion from eggs 249 and 250 (3rd generation) gave typical symptoms, one bird dying on the 3rd day and three others showing typical lesions when killed on the 4th day. The four birds inoculated from the 7th generation showed no symptoms, but when killed on the 7th day three showed definite thickening and inflammation of the tracheal mucosa. The virus was therefore certainly present in the 3rd generation, and probably in the 7th.

The particular strain of virus used appeared to be extremely inconstant in its infectivity for chicks throughout the experiments, and typical fatal lesions were only rarely obtained. It had either lost much of its virulence since isolation, or the available stock of chickens was relatively resistant to its action. For this reason experiments were discontinued after eight egg passages had been obtained. A number of filtration experiments were made which indicated that the virus particles could pass a relatively permeable membrane (0·8μ A.P.S.), but it proved impossible to titrate the virus satisfactorily in chicks, while egg material failed to give infective filtrates even through permeable membranes, and the experiments were abandoned.

SUMMARY.

1. Laryngo-tracheitis virus may be propagated in the chorio-allantoic membrane of the developing egg.

2. The lesions produced in the membrane are primarily due to proliferative and necrotic changes in the ectodermal layer ; proliferating cells frequently show typical intranuclear inclusions similar to those found in the tracheal lesions.

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THE DIFFERENTIATION OF THE VIRUSES OF FOWL PLAGUE AND NEWCASTLE DISEASE: EXPERIMENTS USING THE TECHNIQUE OF CHORIO-ALLANTOIC MEMBRANE INOCULATION OF THE DEVELOPING EGG.

F. M. BURNET AND J. D. FERRY.

From the National Institute for Medical Research, Hampstead, London.

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NEWCASTLE disease of fowls apparently made its first appearance in England in 1926 and was described in 1927 by Doyle, who showed that the infective agent was a filter-passing virus. He differentiated it from fowl plague on the basis of (*a*) the longer incubation period and the distinct clinical syndrome of Newcastle disease, (*b*) the infectivity of the virus for pigeons, (*c*) the absence of any immunity to fowl plague in birds convalescent from and immune to Newcastle disease, and (*d*) the much greater contagiousness of Newcastle disease.

Acute infectious outbreaks amongst fowls apparently due to the same virus have been described from the East Indies (Picard, 1928; Rodier, 1928), Korea and Japan (Konno *et al.*, 1929; Nakamura *et al.*, 1933), India (Cooper, 1931; Kylsamaier, 1931) and Australia. Cooper (1931) showed that the Indian strain "Rhaniket disease" was immunologically similar to strains from Java and the Philippine Islands and to the original Newcastle strain. Nakamura and his collaborators also found that Korean and Japanese strains were immunologically the same as Doyle's virus and sharply distinct from true fowl plague. Manninger (1932), however, regards Newcastle disease as a less acute variety of fowl plague, and claims by repeated passage to have so increased its virulence that it produced a condition clinically and immunologically identical with fowl plague. Picard (1933) in Java also leans toward the view that the viruses differ only in their virulence.

The very high infectivity of Newcastle disease necessitates strict isolation of experimental birds, and this requirement has naturally tended to limit work on the properties of the virus responsible. When it was observed that Newcastle disease virus was highly infective for the developing egg, the use of this technique seemed to promise a much more convenient method of studying the virus than the conventional one involving the use of rigidly-isolated birds. Fowl plague virus is also highly pathogenic for the embryo, so that the two viruses could be compared under precisely similar conditions.

Experience with the method has very largely fulfilled this promise, and has indicated that for a considerable variety of investigations on the acute virus diseases of fowls it is the method of choice. It is naturally incapable of throwing any direct light on the pathology of the natural disease in the bird,

but where, as in most work on the nature of the viruses, the fowl is only needed as an indicator of the presence and amount of virus in a given preparation it can very advantageously be replaced by the developing egg.

The advantages of the method over the usual one may be summarily stated as follows: The embryo is as highly susceptible to the viruses as the chick. Filtrates have been regularly obtained for both viruses which were infective in amounts of 0·03 c.c. of a dilution approximating 1 : 10,000,000 of the infected egg membrane. The embryo is uncontaminated by bacteria, and bacteriologically sterile material may be obtained directly from the lesions for some time after death. The infection is completely confined within the egg, and no cross-infections of experimental or stock birds can occur. The lesions occur in an almost diagrammatically simple tissue in which the pathological histology of the lesion can be readily studied, and finally the expense associated with the purchase, feeding and effective isolation of experimental birds is obviated.

The present communication embodies the results of comparative experiments along these lines with the viruses of fowl plague and Newcastle disease, and deals with (1) the gross and microscopic changes in infected eggs, (2) the size of the virus particles as judged by differential membrane filtration experiments, and (3) the resistance of the viruses to photodynamic inactivation by methylene-blue.

In regard to each of these points characteristic differences between the two viruses have been observed. Taken along with the known clinical and immunological differences, they make it clear that there is no close biological relationship between the agents of the two diseases.

Technique.—The essentials of the technique used for inoculation of the chorio-allantoic membranes have been previously published (Burnet, 1933).

In inoculating considerable numbers of eggs, it has been found much quicker and more satisfactory to make the shell opening by means of a dental engine and suitable abrasive disc. The subsequent steps of the inoculation technique are identical with that previously described, the inoculum being deposited on the chorio-allantoic membrane at the base of an artificial air-sac separating this from the shell membrane. The eggs are incubated at 39° and examined twice daily by transillumination, and, for most experiments with these viruses, opened as soon as possible after death of the embryo. With a little experience one can be fairly certain from the appearances under transillumination whether the embryo is alive or dead. The signs of death are (1) immobility of the embryo, (2) partial or complete disappearance of visible blood-vessels, (3) free mobility of the artificial air-sac and grey moist appearance of the shell membrane in the inoculation window, both due to transudation of fluid between shell membrane and chorio-allantoic. All eggs which were dead when opened were cultured for bacteria, but were almost invariably sterile, provided they had not been dead for more than twenty-four hours.

1. THE PATHOLOGY OF NEWCASTLE DISEASE IN THE DEVELOPING EGG.

The virus used in these studies was obtained through the kindness of Dr. Todd from Dr. T. M. Doyle, in the form of a portion of liver from an infected

fowl, preserved in 50 per cent. glycerine. This was ground up with broth, centrifuged, and the supernatant fluid inoculated into eggs undiluted and diluted 1 : 100. The former eggs became contaminated with bacteria, but those inoculated with the dilute material remained free from contamination and died within 48 hours. All the subsequent virus preparations were derived from an emulsion of the chorio-allantoic membrane from one of these eggs.

When an egg is inoculated with an active virus preparation and incubated at 39–40° C. it usually dies in from 30 to 48 hours, irrespective of the amount of virus administered. With very dilute virus death is sometimes delayed until the third day, but never beyond this, and on one occasion several eggs subsequently shown to contain typical Newcastle disease virus died within 24 hours. This may have been due to the fact that they had been allowed to become cold a few hours before inoculation, but this possibility has not been definitely established. When an egg which has just died is opened the picture presented is, as a rule, immediately characteristic of the disease. The chorio-allantoic membrane which forms the base of the artificial air-sac is abnormally moist, and there is a little free fluid in the angles where it meets the shell membrane. It is thickened with transparent œdema, particularly at the periphery, and may show some degree of cloudiness from cellular infiltration. Scattered over its surface are small irregular flecks of whitish opacity, each only a millimetre or two across and often dendritic in form. These are the areas of ectodermal proliferation which are the characteristic feature of histological preparations. Small petechial hæmorrhages are usually present in the membrane and may form the most obvious feature of the lesion. If the embryo is removed from the shell it will in most instances be found to show numerous sharply-outlined petechiæ, particularly on the neck and along the sides of the ventral aspect of the body.

Histological sections of the chorio-allantoic membrane show the essential features of the lesion to be patchy ectodermal proliferation followed by, or associated with, gross vacuolation of the epithelial cells and eventual necrosis. Œdema and accumulation of inflammatory cells and extravasated red blood-cells in the mesoderm and a variable degree of endodermal proliferation with occasional vacuolation are also usually present. The ectodermal proliferation is the most characteristic feature. This never takes on the massive squamous appearance found in fowl-pox lesions, and usually results in a layer only 3

EXPLANATIONS OF FIGURES.

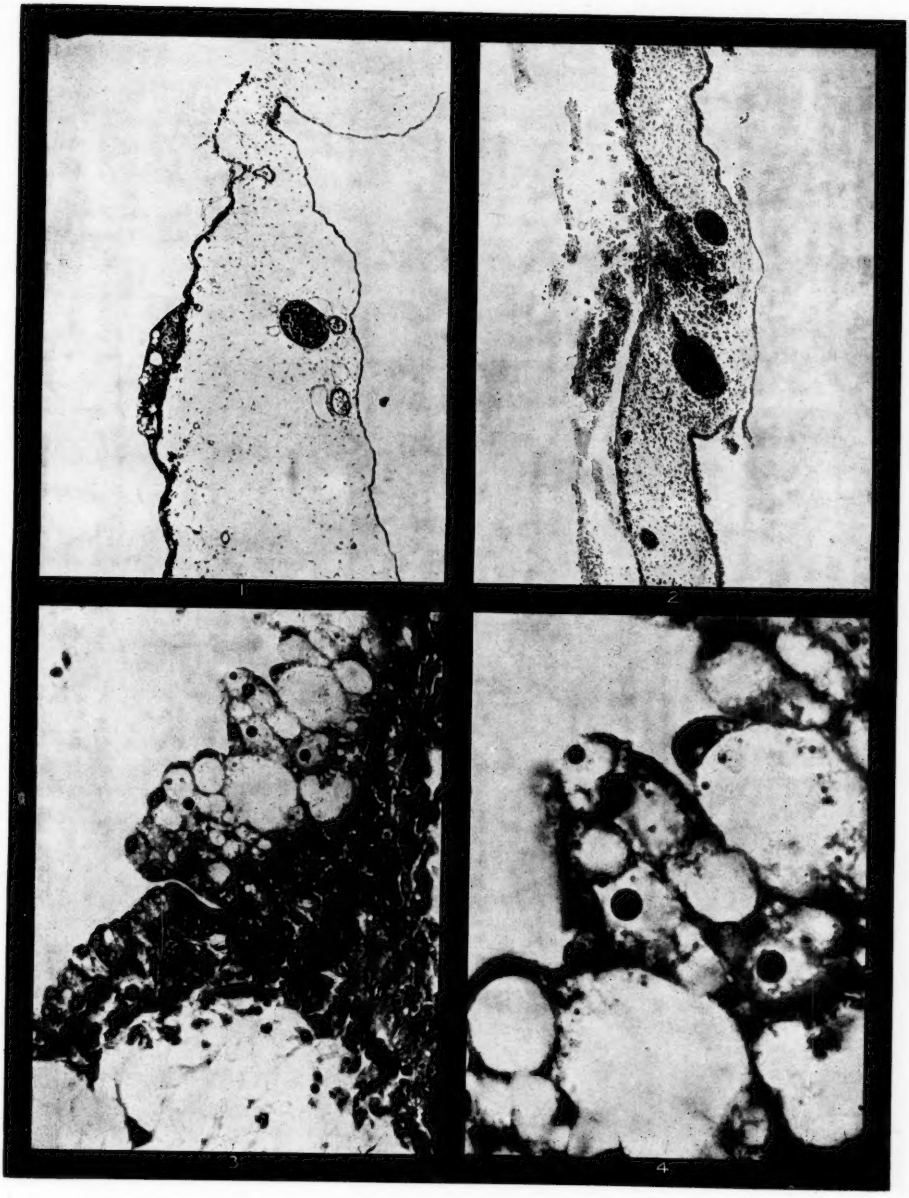
Microphotographs of egg membrane lesions of fowl plague and Newcastle disease viruses.

FIG. 1.—Newcastle disease. Egg 323 × 67. 44-hour lesion from still living egg showing an early plaque of ectodermal proliferation with vacuolation.

FIG. 2.—Newcastle disease. Egg 419 × 67. Lesion from egg a few hours dead. Proliferated and disintegrating ectoderm infiltrated with inflammatory cells; hæmorrhage and cellular infiltration in the mesodermal region.

FIG. 3.—Newcastle disease. Egg 317 × 450. Lesion from egg still living at 44 hours. Proliferated ectodermal epithelium showing vacuolation of outer cells with circular cytoplasmic inclusions lying within the vacuoles.

FIG. 4.—Newcastle disease. A higher magnification (× 1250) of 3, to show several inclusions. Note suggestion of internal structure in inclusion slightly above the centre of the photograph.



Burnet and Ferry.

or 4 cells thick, the outer cells of which are grossly vacuolated, with pale staining nuclei. In many places the vacuoles appear empty, but in localized areas they contain round, sharply-defined acidophil bodies, which appear to be true cytoplasmic inclusions. These vary in size from less than 1μ up to about 5μ in diameter, the largest being found only in the most grossly vacuolated cells. No evidence of granular internal structure can be observed, but some of the largest bodies appear to stain with fuchsin more intensely at one point. When stained with acid fuchsin and differentiated with orange G it is rather easy to decolorize the bodies, which may then show a central red zone with an outer greyish region. Even the smallest of the inclusions will be found surrounded by a clearly visible vacuole, which seems to become progressively larger as the inclusion develops, till we reach the condition shown in Fig. 4. It is usual to find only one inclusion in a cell, but more may be found, and rarely one finds a cell containing 15 or 20 small inclusions. The inclusions have been found in about 50 per cent. of the eggs sectioned. In all cases their distribution has been limited to rather small areas of proliferated epithelium, and only in one case has more than one such area been found in a given section, so that it is probable that a study of a large number of sections from the apparently negative eggs would have revealed their presence.

It is probable that all parts of the infected egg contain the virus. In addition to the membrane lesions which were used as the standard source of virus, liver and brain emulsions proved highly infective. These were removed from carefully washed embryos and were infective in 0·03 c.c. amounts of dilutions, equivalent to approximately 1 : 200,000 and 1 : 2,000,000 of the fresh tissues respectively.

At the conclusion of the filtration experiments two adult hens were inoculated intramuscularly with 0·5 c.c. of (*a*) stock filtrate of 23.10.33, and (*b*) 0·20μ filtrate of 16.x.33 (see Table I), both known to be active in the egg, and both having undergone at least twelve passages through eggs during three months. Fowl *a* showed symptoms on the fifth day after inoculation and was found dead on the eighth day. Fowl *b* showed weakness and a ropy mucous exudate from the mouth on the fourth day, and was obviously moribund, with cyanotic comb and laboured audible breathing, when killed 5 days after inoculation.

The virus, therefore, had maintained its normal virulence through frequent egg passage.

2. FOWL PLAGUE LESIONS IN THE EGG.

The strain of fowl plague used, "Brescia", was obtained from Dr. C. Todd. It was originally isolated by Ascoli, and has been used in numerous investigations by Doerr and his collaborators (1931), and by Elford and Todd (1933). It is of the usual extreme virulence, killing fowls within 48 hours.

When the virus is inoculated on to the chorio-allantoic membrane, death of the embryo occurs within 14 to 18 hours in the great majority of instances. With limiting dilutions death is occasionally delayed until the second day. As is to be expected from the rapidity of its action, the virus produces little in the way of characteristic lesions. The chorio-allantoic membrane is found to be moist, and sometimes shows œdematous thickening ; a few hæmorrhages

are usual, and occasionally slight opacities of the membrane are present. The embryo is usually congested, with occasional diffuse hæmorrhages into the skin, but never shows the multiple, sharply delimited petechiæ characteristic of Newcastle disease. Histologically the membrane usually shows almost complete absence of any ectodermal cells; occasionally one finds a region where slight proliferation has taken place. Œdema is usual, but the degree of inflammatory reaction in the mesodermal layer is highly variable. No appearances suggestive of inclusion bodies have been observed.

As is the case with Newcastle disease, large amounts of virus are present in the embryo liver and brain, as well as in the chorio-allantoic membrane. The virus maintained its full virulence for the fowl through frequent egg passages. Experiments exemplifying this are described below in connection with the photodynamic inactivation of the virus.

A few experiments with a strain of fowl plague of African origin, obtained from Dr. T. M. Doyle, showed no appreciable differences from the strain "Brescia", the eggs being killed in 24 hours with the same absence of any characteristic lesion.

3. FILTRATION STUDIES USING ELFORD'S GRADED COLLODION MEMBRANES.

Some earlier attempts to use virus-containing material from eggs infected with canary-pox virus or infectious laryngo-tracheitis in filtration studies by Elford's technique were unsuccessful owing to the extremely low titres of the stock filtrates obtained. Our first experiments with Newcastle disease, using embryo liver as the source of virus, encountered the same difficulty. The reason for this is not yet clear, but the following technique has been uniformly successful in providing high titre filtrates of both Newcastle disease and fowl plague viruses.

Two or three chorio-allantoic membrane lesions are removed aseptically from eggs recently dead from the infection and washed in 20 or 30 c.c. of sterile broth. They are then finely ground in a mortar with quartz powder and about 2 c.c. of sterile distilled water. The emulsion is placed in a centrifuge tube and the mortar washed out with a few c.c. of broth. This emulsion in diluted broth is centrifuged at moderate speed and the opalescent supernatant then added to 25 c.c. of Hartley's broth. (In most experiments about 2 c.c. of a phage filtrate was also added at this stage. The phage C16 has a particle size only slightly smaller than that of fowl plague virus, and provided a useful control on the uniformity of membranes of about the limiting permeability.) The material is now filtered through a sand and paper pulp filter and then through a permeable membrane (A.P.D.* 0·7–1·0μ) to give a stock filtrate. With both viruses such filtrates have always been infective in a dose of 0·03 c.c. of a 1 : 10,000 dilution, and usually with 1 : 100,000 dilutions.

The filtration studies were carried out with the membranes and by the general technique described by Elford and his collaborators in several previous papers (Elford, 1931; Galloway and Elford, 1931; Elford, 1933). In each filtration 10 c.c. of stock filtrate were filtered through about 1 sq. cm. area of membrane at 1 atmosphere positive pressure. For the final experiment with

* A.P.D. = Average pore diameter.

TABLE I.—*Filtration Experiments with Newcastle Disease Virus using Egg Material.*

Date.	Source of virus.	Stock filtrate and titre.	Secondary filtrations. Membrane A.P.D.	Infectivity.	Phage content.
24.viii.33	Embryo liver	0·55μ Inactive	..	..	..
31.viii.33	Washed C-A membranes	0·78μ 1 : 10,000, + 2 d.	..	..	..
		1 : 100,000, + 3 d.	..	..	..
4.ix.33	,, ,,	0·78μ 1 : 10,000, + 2 d.	0·35μ	Undil., + 2 d., + 2 d.	80%
			0·20μ	,, S, S	0·12%
11.ix.33	,, ,,	0·78μ 1 : 10,000, + 2 d.	0·30μ	1 : 100, + 2 d.	50%
		1 : 100,000, S		1 : 1000, S	
			0·20μ	Undil., + 2 d., + 2 d.	24%
14.ix.33	,, ,,	1·0μ 1 : 100,000, + 3 d.	0·20μ	,, + 3 d., S	0·14%
			0·25μ	,, + 2 d., + 3 d.	Not tested.
16.x.33	,, ,,	0·75μ 1 : 100,000, + 2 d.	0·20μ	,, + 2 d.	1·6%
				1 : 10, + 3 d.	
				1 : 100, S	
			0·16μ	Undil., S, S	0 004%
23.x.33	,, ,,	0·76μ 1 : 100,000, + 2 d.	0·16μ	,, S, S (two 10 c.c. samples)	0·01%

Notes.—The thicknesses of filter membranes ranged from 0·11 mm. to 0·15 mm. + = death of embryo within period shown in days (d.). S = survival more than 3 days without lesions. The phage content is expressed as the percentage of plaques produced relative to those obtained from the stock filtrate.

TABLE II.—*Filtration Experiments with Fowl Plague Virus using Egg Material.*

Date.	Source of virus.	Stock filtrate and titre.	Secondary filtrations. Membrane A.P.S.	Infectivity.
7.ix.33	Washed C-A membranes	0·78μ 1 : 10,000, + 1 d.	0·20μ	Undil., + d.
				1 : 100, + 1 d.
			0·13μ	Undil., S, S
16.x.33	,, ,,	0·75μ 1 : 10,000, + 2 d.	0·20μ	,, + 1 d.
			0·16μ	,, + 1 d., + 1 d.
				,, + 1 d.
22.x.33	,, ,,	0·76μ 1 : 100,000, + 1 d.	0·16μ	1 : 100, + 2 d.
				1 : 1000, S

The thicknesses of filter membranes ranged from 0·11 mm. to 0·21 mm. Abbreviations as in Table I.

Newcastle disease 20 c.c. were filtered through a 0·16μ A.P.D. membrane and two 10 c.c. samples tested separately. All the filtrates were tested for their virus content by the inoculation of from 0·025–0·03 c.c. on to the chorio-allantoic membranes of developing eggs, and when phage had been added this was titrated by the usual plaque-counting method. The results with Newcastle disease virus are shown in Table I, and a smaller series of experiments with fowl plague virus in Table II. It will be seen that the particle size determined for fowl plague virus by the present technique is the same as that found by Elford and Todd (1933) with the usual test by inoculation of half-grown chicks. The virus of Newcastle disease is definitely larger than that of fowl plague, although the difference is not great. Fowl plague virus regularly passes 0·16μ A.P.D. membranes, which retain Newcastle disease virus. The latter is also largely held back by 0·20μ membranes, the results with which are irregular, suggesting that the end-point is not far below this value. The variation in the percentage phage content of the secondary filtrates through 0·20μ membranes (except that of 11.ix.33, which is inexplicably high) corresponds roughly to differences in the phage concentration in the stock filtrates, and may be due also to variation in the degree of pore-blocking by the virus particles. In taking 0·16μ as the end-point, the difference between the sizes of the two viruses is certainly not over-estimated. Using Elford's (1933) formula, the particle size of Newcastle disease virus is 80–120mμ as compared with 60–90mμ for fowl plague virus.

4. PHOTODYNAMIC INACTIVATION BY METHYLENE-BLUE.

Perdrau and Todd (1933) found that fowl plague virus in the form of a filtrate from infected plasma was highly sensitive to inactivation by methylene-blue in the presence of light. A number of experiments have been made to compare the resistance of fowl plague and Newcastle disease viruses to such photo-dynamic inactivation.

The material used has been throughout in the form of stock membrane filtrates in broth made as described above from egg-membrane lesions. The conditions of inactivation were kept constant, and only the time of exposure varied in different experiments. The final concentration of methylene-blue was 1 : 50,000, and the mixture was exposed in a layer 2 mm. deep, placed 15 cm. below a 300 c.p. filament lamp. Heating of the virus was avoided by interposing a glass dish filled to a depth of about 3 cm. with water between the lamp and the virus mixture. A constant flow of cool tap-water was maintained through the dish during each irradiation.

Under these conditions Newcastle disease virus proved capable of resisting an exposure of 30 minutes. After this period a stock filtrate with an initial titre of 1 : 100,000 was active when diluted 1 : 100 but not at 1 : 1000. Fowl plague virus was considerably less resistant, being reduced to a titre of 1 : 10 from 1 : 100,000 after 10 minutes' irradiation. Even so, it is evident that the egg virus filtrates are not so readily inactivated as the filtrates used by Perdrau and Todd, the infectivity of which was destroyed by 5 minutes' irradiation with a 100 c.p. light at 20 cm. In order to consider the possibility that the egg might be a more sensitive detector of active virus than the fowl, one

experiment was done in parallel, the irradiated mixtures being inoculated in 0·025 c.c. quantities into eggs and 0·5 c.c. being given intramuscularly to adult fowls. Material irradiated for 5 to 10 minutes gave the classical symptoms in each host, killing the egg within 24 hours and the bird within 48 hours. The filtrate irradiated for 20 minutes killed the egg within the usual period, whereas the hen appeared ill on the third day and was found dead about 90 hours after inoculation.

The difference in the rate of inactivation of the two viruses may be used to separate them in mixtures. A mixture containing equal parts of highly active filtrates of fowl plague and Newcastle disease lesions was irradiated under standard conditions for 10 minutes. Eggs inoculated with the undiluted irradiated material died in 24 hours (fowl plague); those inoculated with a 1 : 100 dilution died after 2 days and showed the typical proliferative lesions of Newcastle disease. The whole series of methylene-blue irradiation experiments are summarized in Table III.

TABLE III.—*Summary of Methylene-blue Inactivation Experiments.*

Times of irradiation.	Number of experiments.	Dilution of irradiated material. Undiluted.	1 : 10.	1 : 100.	1 : 1000.
Fowl plague :					
5 minutes	4	+, +, +, +*	+	..	..
10 ,,	3	+, +, +*	+, —	—, —	..
20 ,,	2	+*, —	—	..	..
Newcastle disease :					
30 minutes	3	+, +, +	+, +	+, —	—
Mixed viruses :					
10 minutes	1	+ F.P.	..	+ N.D.	..

Conditions of irradiation identical apart from time (see text). + = death of embryo with typical lesions; — = survival of embryo without lesions; +* = positive fowl plague death in both egg and fowl; F.P. = fowl plague; N.D. = Newcastle disease.

SUMMARY.

1. Newcastle disease and fowl plague viruses are highly infective for the developing egg, and the use of this technique for their investigation offers a number of advantages.

2. Newcastle disease virus produces a characteristic lesion in the chorio-allantoic membrane, in which cytoplasmic inclusions can be demonstrated histologically.

3. Comparative filtration studies with egg material indicate that Newcastle disease virus is larger (80–120 mμ) than fowl plague virus (60–90mμ).

4. Newcastle disease virus is more resistant to photodynamic inactivation by methylene-blue than fowl plague virus.

5. These differences in conjunction with the known clinical and immunological differences point to the complete ætiological independence of the two diseases.

We are indebted to Dr. T. M. Doyle and Dr. C. Todd for providing the viruses used in this investigation.

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ATTEMPTS TO LOCATE THE SITE OF ANTIBODY PRODUCTION.

G. A. H. BUTTLE, M.R.C.S., L.R.C.P.

From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

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MANY investigators have attempted to locate the site of antibody production; some have considered that the "reticulo-endothelial system" was responsible, others that all tissues contributed to the response. It has already been shown that the removal of the spleen does not affect the antibody response (Glenny and Sudmersen, 1921).

Topley (1930) found that spleen mush from an animal which was about to develop agglutinins caused a rise in titre when injected into a normal animal, and concluded that the spleen was concerned in the elaboration of antibody or some intermediate product.

It has been reported that tissue cultures will produce antibodies. Thus Carrel and Ingebrigtsen (1912) showed that a tissue culture of guinea-pig bone-marrow and lymph-nodes to which sheep cells were added would produce a hæmolysin. Kurt Meyer and Lowenthal (1927) found that tissue cultures of rabbit's spleen, lymph-glands and "milk spots of the peritoneum" taken from an animal three days after an injection of paratyphoid B organisms produced agglutinin to the organism. No attempt was made to differentiate between different types of tissue.

Attempts have been made to extract antibody from the tissues of animals whose antibody titre is increasing. Thus Cannon and Sullivan (1932), using

animals immunized by intracutaneous injections of paratyphoid B found that they could extract agglutinins from the skin in the vicinity of the injection in a greater concentration than from the skin of the opposite side. They concluded that the antibody was produced locally in the skin in this case.

I have attempted to extract diphtheria antitoxin from the tissues of guinea-pigs whose titre was increasing as the result of a secondary stimulus, but in no case was it possible to show a larger quantity of antitoxin in the tissues than in the blood at the same time; probably the local effect in this case is not so marked as with the agglutinins, as the antigen is freely diffusible throughout the tissues.

It seemed worth while ascertaining whether certain organs played a predominant part in the production of antibody. It was necessary to obtain a quick rise in an antibody which it was possible to estimate accurately, in order to detect a difference during the short time that the experiment lasted. Diphtheria antitoxin can be titrated more accurately than any other. An animal which has been immunized with a previous injection will give a large and quick response to a second injection of toxoid—the secondary stimulus of Glenny and Sudmersen (1921).

The response to a secondary stimulus is greater if a long interval has elapsed after the primary stimulus—the response after three months is much greater than that after three weeks (between two- and ten-fold). The explanation of this would seem to be that in the intervening period the antitoxin in the blood gradually falls after reaching a maximum at four or five weeks after the primary stimulus; probably the antitoxin in the tissues does not fall so rapidly. Thus when the secondary stimulus is given soon after the primary, the antitoxic content of the blood is relatively high, and a large amount of toxoid (of the secondary stimulus) is neutralized by the antitoxin in the blood, and does not reach the other tissues, while, if it is given at a later time, a greater amount of toxoid reaches the tissues where presumably the antitoxin is formed. In this series of experiments an interval of at least three months was allowed after the primary stimulus.

Primary stimulus.—The rabbits were injected subcutaneously with a small dose of 2 per cent. alum diphtheria toxoid, 24 Lf units per c.c.; 5 c.c. of a 1/10 dilution was used. The response to such a stimulus was found to be more uniform than that to plain toxoid unmixed with alum; one variable, viz. the rate at which the animal can eliminate the plain toxoid, is to some extent eliminated (Glenny, Buttle and Stevens, 1931). The animals were bled at the end of one or two months, and those of similar values grouped together for each experiment, it being found that the secondary response of such a group was more uniform than if the animals were not so selected. The animals were then used three to four months after the stimulus.

Titration of antitoxin.—This was done by the guinea-pig intracutaneous method. Differences of 10 per cent. were looked for in the case of the shorter experiments.

EXPERIMENTS.

As the blood contains a vastly greater amount of antitoxin than any other tissue of the immune animal, it was considered possible that the blood might

be producing the antitoxin. In order to determine this, the blood of an animal on the third day after a secondary stimulus was as far as possible substituted by normal blood. The animals were anæsthetized with ether, and 60–70 c.c. of blood removed from the carotid artery until there was alteration in the breathing. Normal blood diluted with an equal volume of saline, rendered incoagulable with hirudin and heated to 37° C. was then run into the femoral vein, and successive quantities of 40–60 c.c. run out of the carotid artery. Towards the end of the transfusion undiluted blood was run into the vein and the volume adjusted, so that an equal quantity of fluid had been put in and

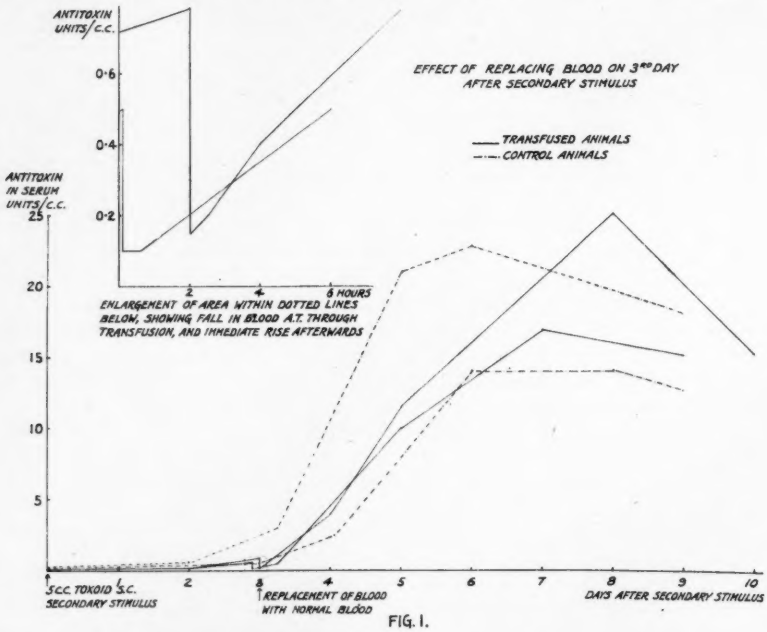

FIG. 1.

taken out. In all, 200–300 c.c. of fluid was used for each transfusion, and thereby 4/5 of the circulating blood was substituted by normal rabbit blood; this was determined by the fact that the antitoxin titre of the blood fell from 0·75 units per c.c. to 0·15 units per c.c. in one case, and 0·5 to 0·1 in the other.

Fig. 1 shows that the rise in antitoxin titre in the transfused animals started again as soon as the transfusion was finished, and that the response was not in any way different from that of the untransfused controls. The figure shows results on two control rabbits and two transfused rabbits, all injected with alum toxoid three months previously. A secondary stimulus of 5 c.c. of toxoid was given, and the graphs show the course of the serum antitoxin after this. The transfusion was performed three days after the secondary stimulus, and the inset shows on a larger scale the course of the antitoxin in the first few hours after the transfusion.

It may be assumed, therefore, that the blood does not produce the antitoxin, but merely acts as the vehicle in which it is carried around the body after it has been produced by other tissues.

It is of some interest to note that no appreciable quantity (only about 4 units, or the equivalent of 7 c.c. of serum) of antitoxin was "washed out" of the tissues during the transfusion, the amount obtained in the washings being approximately equal to the quantity in the blood at the beginning of the transfusion. (See antitoxin balance-sheet, Appendix.) It would appear,

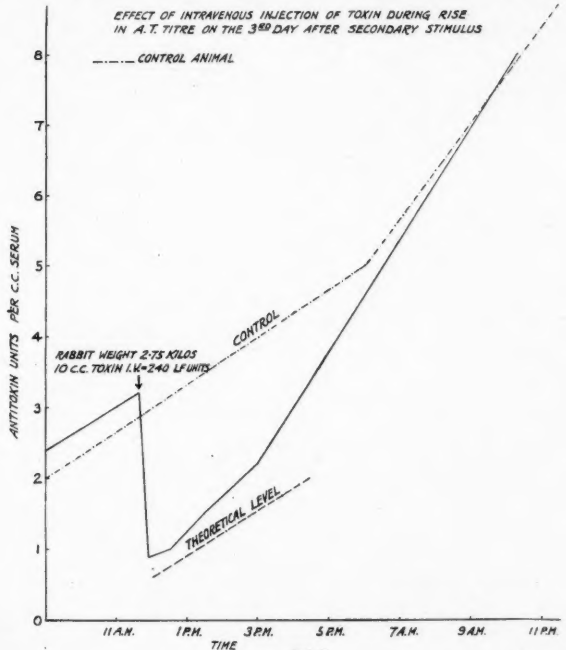

FIG. 2.

therefore, that there is no great "store" of antitoxin in the animal capable of being washed out during the rise in titre other than the antitoxin in the blood. In this connection the result of an intravenous injection of toxin into an animal during the rise in titre is of some interest. Fig. 2 shows this effect. It will be seen that the fall in the titre of the blood-serum after an intravenous injection is equal to the fall expected from the Lf value of the toxin (within the error of the experiment). So that all the antitoxin available for neutralizing the injected toxin is in the blood, and no tissue antitoxin helps to neutralize it.

The fact that the antitoxin is not produced in the blood was shown in another way. An animal was bled on the third day after a secondary stimulus, and the blood injected into a normal animal. An immune animal was selected with a low antitoxin value under 1/1000, a secondary stimulus of 10 c.c. of

toxoid was given, and on the third day 35 c.c. of hirudinized blood from this animal was put into a normal animal. The normal animal did not produce any antitoxin, whereas the immune animal went up to ½ unit per c.c. of serum on the sixth day. The animals weighed 3 kilos, so that about one-fifth of the blood of the immune animal was put into the normal animal, whose blood had under 1/1000 of a unit per c.c., three days after the transfusion at the time when the immune animal had ½ unit per c.c. Under these conditions, therefore, the blood does not produce as much as 10/1000 or 1/100 of the antitoxin produced by the whole animal.

As the "reticulo-endothelial" system is commonly supposed to be the

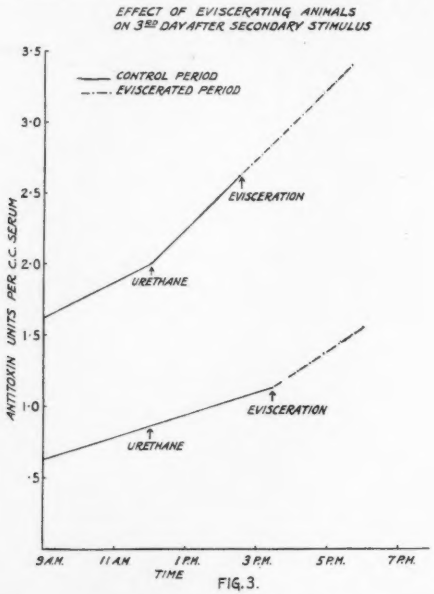

FIG. 3.

source of antibody, the effect of removal of the liver and spleen was tried. On the third day after a secondary stimulus the rabbits were eviscerated under complete anæsthesia with urethane, which was maintained until "death". The evisceration was performed as follows: The abdomen was opened and the superior mesenteric and cœliac axis vessels tied off, also a small branch of the lower aorta supplying the colon; the portal vein was then tied off. Ligatures were put round the rectum and œsophagus, and also around the base of the lobes of the liver where the hepatic veins leave them. First the gut and then the liver was removed without loss of blood, and the animal was then sewn up. An intravenous injection of glucose, ½ gr. per kilo, was given after two hours. The results are shown in Fig. 3. It will be seen that the rate of increase of antitoxic titre in the animals is not significantly different before and after evisceration.

In calculating the antitoxin values of the blood of these animals, a correction

is applied for the dilution of the blood during the eviscerated period. This was determined by finding the percentage of solids in the plasma before and after the experiment. The dilution in each of these animals was 5 per cent.

Fig. 4 shows the results with two animals completely anæsthetized with urethane during the whole observation. They had their skin removed on the third day after a secondary stimulus; they were kept in the incubator at 37° C., and covered with wool damped in saline. It was found impossible to keep the animals for more than a few hours in this condition, but it appears that the removal of the skin does not materially affect the antitoxin response.

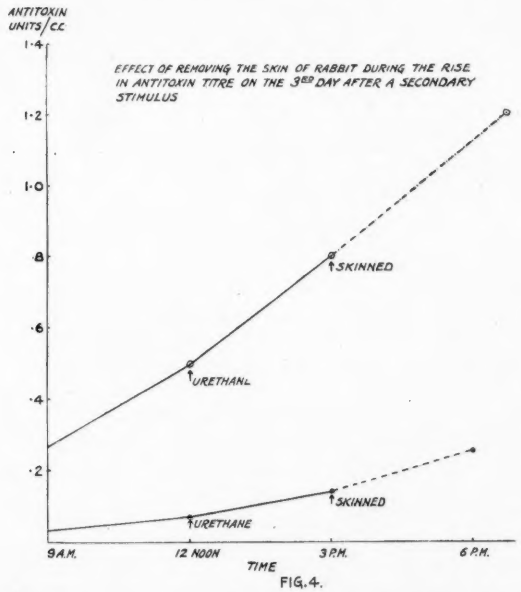

FIG. 4.

Here, again, corrections were applied for changes in the concentration of the blood, there being a dilution after the fluid in which the urethane was dissolved was absorbed, and later a concentration when the skin was removed.

CONCLUSION.

The blood, liver and spleen and the skin are not predominantly responsible for the production of antibodies; it appears probable that all tissues take part in the production of antibodies, and as these organs form a relatively small part of the total mass of the animal, the effect of their removal is not obvious.

SUMMARY.

Substitution of the blood with normal blood, removal of the liver and spleen and removal of the skin do not affect the rate of production of diphtheria antitoxin in rabbits.

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APPENDIX.

ANTITOXIN BALANCE-SHEET FOR A TRANSFUSION EXPERIMENT.

Rabbit No. 104 : Weight of rabbit = 3·32 kilos.

Probable content of blood-serum $= \frac{3320}{100} \times 2{\cdot}7 = 90$ c.c.*

First bleeding 65 c.c. blood = 39 c.c. serum.

Therefore serum left in animal after first bleeding = 90 — 39 = 51 c.c.

Titre of antitoxin in serum at the start = 0·56 units per c.c.

Therefore total antitoxin in the serum at the end of first bleeding = 51 × 0·61 units = 31·1 units.

Second bleeding 50 c.c. of diluted blood = 40 c.c. plasma ; titre 0·42 units per c.c.

Therefore total antitoxin removed in second bleeding = 16·8 units.

Third, fourth and fifth bleeding total = 152 c.c. of diluted blood = 122 c.c. of diluted plasma ; titre 0·15 units per c.c.

Therefore total antitoxin removed in third, fourth and fifth bleedings = 18·3 units.

At the end of transfusion animals contain 90 c.c. serum ; titre 0·115 units per c.c.

Therefore total antitoxin in animal at end of transfusion = 90 × 0·115 = 10·3 units.

Balance.—Antitoxin in animal at end of first bleeding = 31·3 units.

Antitoxin removed in second to fifth bleedings = 16·8 + 18·3 = 35·1 units.

It would seem that 35·1 units of antitoxin have been washed out of the animal, whereas the estimated amount of antitoxin in the blood-serum of the animal at the beginning of the process was only 31·1 units. The serum volume could not be determined directly, but had to be deduced from that of rabbits of similar weight, so that the difference is within the error of the experiment.

* The proportion of serum volume to body-weight, 2·7 per cent., was found by injecting intravenously a known quantity of diphtheria antitoxin into rabbits of a similar weight ; there was no direct estimation of the blood volume of the rabbit.